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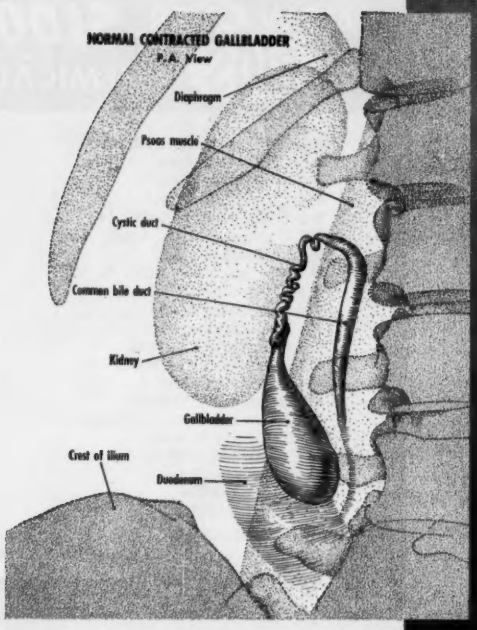

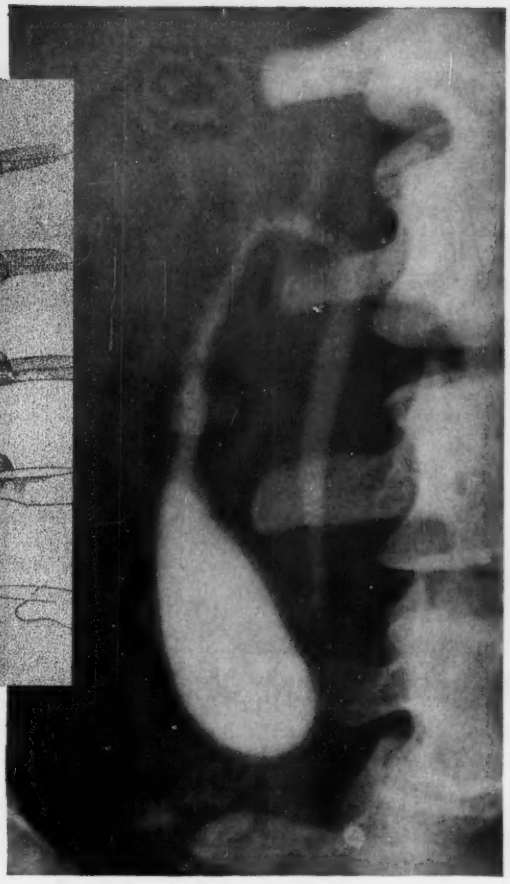

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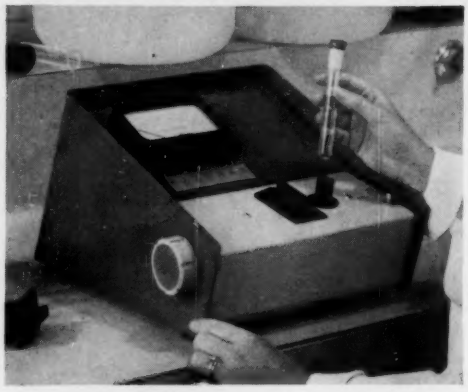

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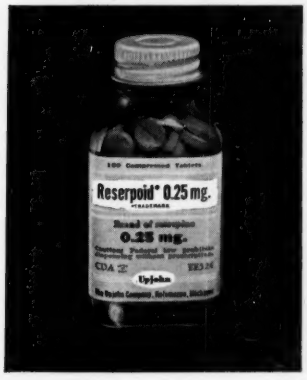

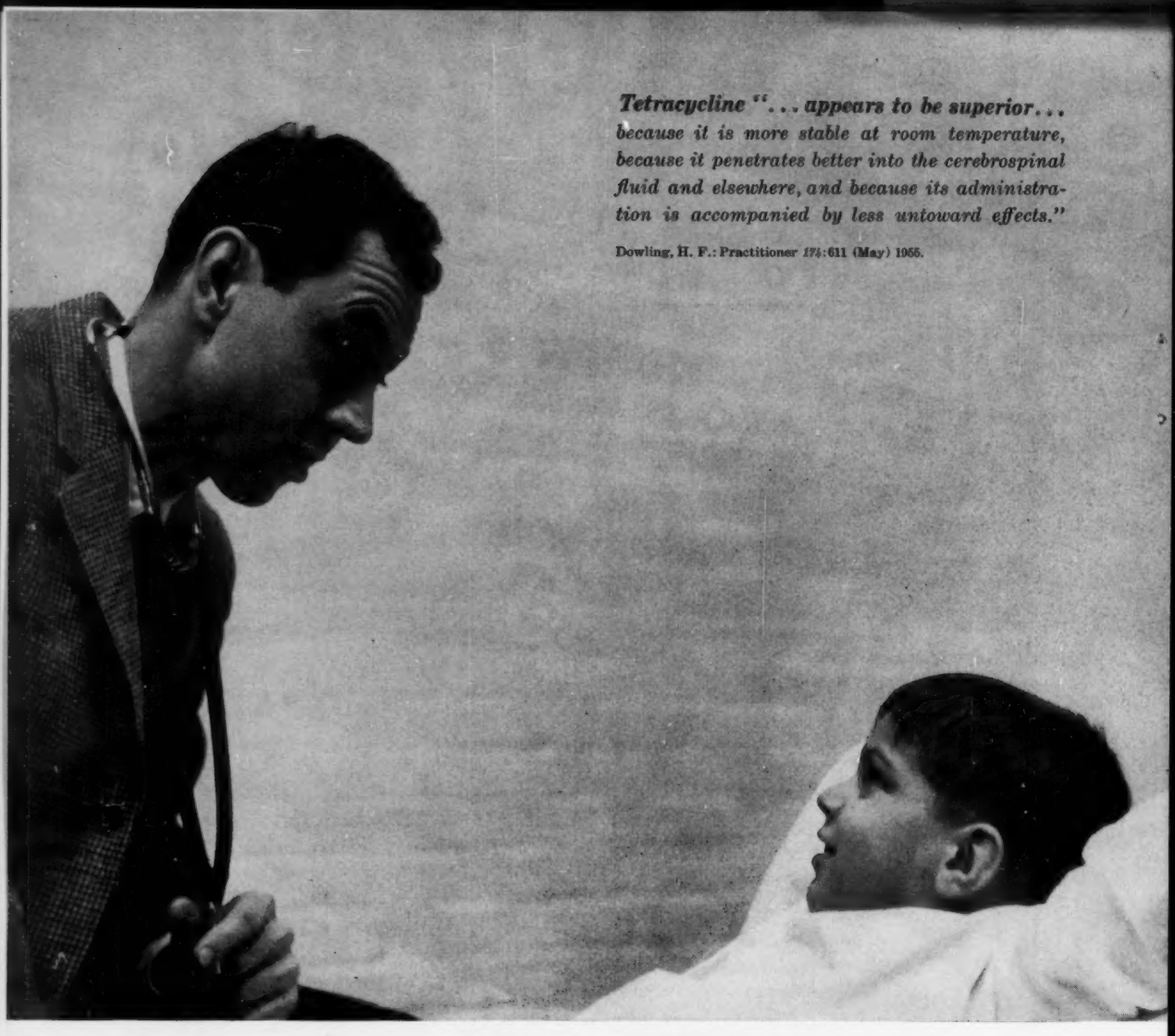

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THE DISPLACEMENT OF SERUM WATER BY THE LIPIDS OF HYPERLIPEMIC SERUM. A NEW METHOD FOR THE RAPID DETERMINATION OF SERUM WATER[1]

By MARGARET J. ALBRINK, PAULINE M. HALD, EVELYN B. MAN, AND JOHN P. PETERS

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(Submitted for publication April 1, 1955; accepted June 1, 1955)

In the course of studies on lactescence of serum, it was found that the insoluble lipids displaced serum water to a degree which was occasionally great enough to be of considerable practical importance. The immediate consequence of this displacement was the finding of spuriously low concentrations of water-soluble components of serum, although their concentrations were normal when repeated on sera from which the insoluble lipids had been removed by ultracentrifugation.

These observations clearly showed the need for a simple, rapid method for determination of serum water that would be applicable to sera with a high concentration of lipids. The method using Karl Fischer reagent (1) has been adapted for the rapid determination of serum water (2) but it was not known whether this would be applicable to highly lactescent sera. A new method based on freezing point depression meets the requirements stipulated and is reported herein. It is compared with the gravimetric method and the serum protein method of estimating serum water. The dilution effect of lipids on serum has been evaluated.[2]

METHODS

A. Chemical methods:

Lipids are reported as total fatty acids released by the hydrolysis of the lipid extract (4). Total proteins were determined by the Kjeldahl method when the serum was lactescent, and by the biuret method when it was clear. Chloride was determined by a micro modification of the Volhard method (5). A flame photometer was employed for the determination of sodium (6).

B. Ultracentrifugal studies:

Lactescent sera were centrifuged at 18,000 r.p.m. for one hour. The clear subnatant fluid was removed as previously described (7). The original and subnatant fluids were subjected to analysis.

C. Gravimetric estimation of serum water:

The water was evaporated and the weight loss used to calculate the serum water in grams per 100 ml. of serum (8).

D. Calculation of serum water from serum protein:

Serum water was calculated from the following formula, the derivation of which is explained elsewhere (8):

$$W_s = 98.5 - 0.745\ P_s$$

in which W_s = serum water in gm. per 100 ml. of serum, and P_s = serum protein in gm. per 100 ml.

E. Osmometric method for estimation of serum water:

Theory: The osmotic pressure of serum is determined before and after the addition of a known amount of dry sodium chloride designed approximately to double the osmolarity of serum. The only change taking place upon this addition is an increase in the molal concentration of sodium chloride. Since the osmotic pressure of a solution is determined by the molal concentration of solute particles, the exact molal concentration of the added sodium chloride can be calculated from the increase it causes in the osmotic pressure of serum. Knowing both the absolute amount and the molal concentration of the added salt, the volume of water in which it was dissolved can then be calculated.

A freezing point osmometer was used for the determination of osmotic pressure. Although osmotic pressure is not actually measured, advantage is taken of the proportionality between osmotic pressure and freezing point depression in such a way that the results are read directly as milliosmols per liter.

Reagents: 1. 0.161 molar sodium chloride solution. Dilute 9.404 gm. dry sodium chloride (reagent grade) to one liter.
2. Double distilled water.

Special equipment: A Fiske Associates freezing-point osmometer was used for the determinations of osmolarity.

Procedure: To prepare the dry sodium chloride exactly 2 ml. of the sodium chloride solution were pipetted into test tubes to be used later in the osmometer. These were brought to dryness in an oven at 98° C. and subsequently stored in a desiccator until ready for use. The addition of exactly 2 ml. of double-distilled water to the dry salt results in a 0.161 molal solution of sodium chlo-

[1] This study was aided in part by a Grant from the National Institutes of Health, and an Institutional Grant from the American Cancer Society.

[2] Part of this study has been presented in abstract form (3).

TABLE I

Electrolytes of lactescent fluids before and after removal of insoluble lipids by ultracentrifugation

Patient* Age Sex	Date		Total fatty acids	Na	Cl	CO_2	Serum water	Comment
			mEq./L.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*gm./100 ml.*	
B82609	8/17/53	Original		102.5	74.5	13.2	72.7	Diabetic acidosis, mild
18 M	2 PM	Subnatant		135.0	102.0		90.2	
	9 PM	Original	526	103.0		16.3	72.5	
		Subnatant	33	134.5	100.0		91.3	
	8/18/53	Original	401	115.5	80.2	19.7	78.0	
		Subnatant	31	135.9	95.5			
39-38-38	8/21/53	Original	187	120.0	102.0	9.2	86.7	Nephrosis
3 M		Subnatant	41	140.0	112.0			
Yan	11/11/53	Original	226	134.8			83.5	Essential hyperlipemia
45 M		Subnatant	29	148.5			90.6	
B60586	11/17/53	Original	51	131.2			89.5	Diabetic acidosis
31 F		Subnatant	30	142.4				
Chyle I	9/30/53	Original	102	143.0			91.9	
		Subnatant	10	149.0			94.7	
Chyle II	9/30/53	Original		132.0			83.3	
		Subnatant		149.0				
C63179	12/29/53	Original	46	135.0			89.3	Diabetic acidosis
35 F		Subnatant	31	139.0			90.2	
B78936	1/8/54	Original	133				88.2	Essential hyperlipemia
50 F		Subnatant	27				91.2	

* Entries are sera unless otherwise stated.

ride containing exactly 300 mOsm. per L. This may be used for the first standard required for the procedure below. (The addition of 2 ml. of water to salt prepared by evaporating 4 ml. of the saline solution results in a solution having an osmolarity of exactly 589.3 mOsm. per L. which may be used for the second standard.)

The osmometer must have been calibrated accurately initially. In addition it must be checked at the beginning and end of each run with two known standards, one having about the osmolarity of serum (300 mOsm. per L.), and the second about double this concentration. These may be prepared as indicated in the previous paragraph.

The osmotic pressure of native serum is first measured using 2 ml. of serum. Duplicate readings are taken. Readings usually check within 1 mOsm.

For the second measurement, exactly 2 ml. of the serum, which may include the thawed serum used in the first determination, are pipetted into a test tube containing the dry saline from 2 ml. of 0.161 molar sodium chloride solution. The tube is gently rotated to insure complete solution, and the osmolarity again measured. If necessary the first and second readings are corrected for any error noted in the readings of the first and second standards, respectively. The procedure is extremely simple, requiring about 8 minutes for the two sets of readings.

The serum water in ml. per 100 ml. serum is calculated from the following formula:

$$(R_2 - R_1)x = A$$

in which R_1 is the reading of the serum, R_2 is the reading of serum with added salt, x is the per cent water by volume, and A is the added sodium chloride expressed as net effective concentration in milliosmols per liter. When the dry sodium chloride is prepared as described above, A = 289.3.[8]

[8] If the activity coefficient of sodium chloride were 100 per cent at the concentration expected in this procedure, each millimol would produce 2 milliosmols. However, it is necessary to make a correction for the decreased ionic activity brought about by increasing concentration. At the osmolarity of serum, about 300 mOsm. per L., the activity coefficient of sodium chloride is 93.2 per cent, and at double the concentration it is 91.5 per cent, a decrease of 1.7 per cent (9). Moreover, the added salt also depresses the ionic activity of the original serum (assuming sodium chloride to be the most important determinant of osmotic pressure in serum) by the same amount, 1.7 per cent. Rather than correct the osmolarity of the original serum, a double correction is made for the added salt. The addition to serum of 161 millimols of sodium chloride is equivalent to $161 \times 2[93.2 - 2(1.7)]$, or 289.3 milliosmols.

Rather than test the method exclusively on normal sera in which little variation in serum water or lipids might be expected, miscellaneous sera from a variety of patients were selected to insure a wide range of water content and to exclude the possibility that various abnormalities of serum might interfere with the method.

RESULTS AND COMMENTS

A. Ultracentrifuge studies:

The most striking change in serum electrolytes following centrifugation occurred in the following case. A 16-year old diabetic boy was admitted in mild diabetic acidosis. For at least four months his diabetes had been poorly controlled and he had noticed a rash. He was slightly drowsy but otherwise he felt well. Examination revealed an extremely thin but healthy-looking boy who displayed the typical rash of xanthoma diabeticorum on the extensor surfaces of his arms and legs. The only other positive finding was lipemia retinalis. In view of the mildness of his illness it was startling to find that his serum sodium was only 102.5 mEq. per L., and the chloride 74.5 mEq. per L. The serum was markedly lactescent. After removal of the insoluble lipids by ultracentrifugation the electrolytes of the clear subnatant fluid were perfectly normal.

TABLE II

Serum water determined by three different methods: its relationship to concentration of proteins, lipids, and sodium in serum

Patient†	Date	Total fatty acids	Total proteins	Sodium		Osmotic pressure	Water			Specific gravity	Comment
				mEq./L. of serum	*mEq./L.* serum water		Grav.	Osm.	Protein		
		mEq./L.	*gm. %*			*mOsm./L.*	*gm. %*	*ml. %*	*gm. %*		
40–11–26	1/13/54	48.4	6.5	137	152	285	90.3	89.3	93.7	1.016	Obstructive jaundice
Serum A	2/19/54					314	92.7	92.5		1.028	Normal serum
Serum B	2/19/54					306	92.7	92.4		1.020	Normal serum
Serum C	2/19/54					315	92.4	91.5		1.019	Normal serum
Serum D	2/19/54					285	92.2	92.0		1.022	Normal serum
Ascitic fluid	1/26/54	1.5				285	98.7	99.7		1.005	
B82609*	8/17/53			102	141		72.7			0.999	Diabetic acidosis
	8/17/53	526		103	142		72.5			0.958	Lactescent, blood sugar 392
	8/18/53	401	5.8	115	147		78.0		94.2	1.002	Blood sugar 241
	8/19/53	231				297	78.5	78.0		0.976	Blood sugar 278
	8/21/53						86.3	85.6		0.951	
40–21–66	2/4/54	19	5.1	132	141	322	94.0	93.0	94.7	1.013	NPN 179 mg. %
39–38–38*	8/21/53	187	3.3	120	138		86.7		96.0	0.982	Lactescent
Chyle I*	9/30/53	102		143	156		91.9				
Chyle II*	9/30/53						83.8	84.0			
Yan*	11/11/53	226	8	135	162	287	83.5	83.8	92.5	1.010	Lactescent
B60586*	11/17/53	51		131	146		89.5			1.018	Lactescent, blood sugar 118
40–53–29	4/1/54	31	8				91.8		92.5	1.019	Renal disease
34629	3/19/54			129	139	311	92.8	92.5		1.019	Diabetic
28039	3/4/54					337	92.4	91.8		1.024	Blood sugar 341
Pooled serum	9/7/54	13.6		141	152	290	93.0	94.0		1.016	
Diluted serum	9/7/54						96.5	96.5			
A91724	9/28/54	9.8	7.2	145	157	284	92.5	93.0	93.1	1.015	Blood sugar 121
41–56–58	9/30/54	49	6.9			287	91.0		93.4	1.014	Icteric
39–35–61	9/30/54	15	8.1			297	91.3	91.8	92.4	1.017	Biliary cirrhosis
A58066	9/15/54	7.9	5.9	144	154	300	93.4	93.6	94.1	1.014	
A72286	10/8/54	11.5	6.2	141	152	287	92.9	93.2	93.9	1.011	Hyperthyroidism
41–33–53	10/8/54	30.2	5.6			320	91.7	92.4	94.3	1.001	NPN 91 mg. %, slightly lactescent
Kra	10/4/54	19.2	6.9			295	92.2	92.5	93.4		
A83882	11/12/54	13.2	7.0			295		93.0	93.3		Diabetic ketosis
41–04–06	11/10/54	8.8	2.9	132	138	267	95.8	96.8	96.3	1.006	Hypoproteinemia
	12/1/54		3.0	132	137	273	96.2	95.5	96.3	1.012	
41–69–79	10/29/54	32.9				306	90.4	91.6		1.034	Slightly lactescent
41–75–65	11/2/54		3.8	112	117	244	95.8	96.8	95.7	1.014	Hypoproteinemia
Pooled serum	11/29/54		5.9			285	93.7	93.5	94.1	1.017	
C63179	12/29/53	46.6		135	151	319	89.3			1.021	Diabetic acidosis, lactescent

* Ultracentrifugal studies shown in Table I.
† Entries are sera unless otherwise stated.

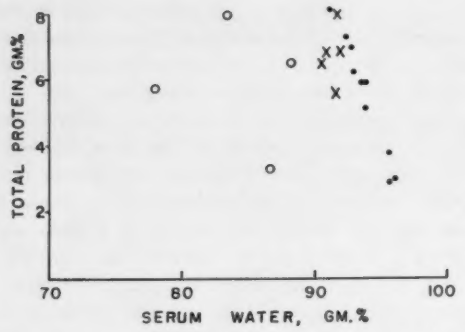

FIG. 1. RELATIONSHIP BETWEEN THE CONCENTRATION OF PROTEIN AND WATER IN SERA HAVING VARYING CONCENTRATIONS OF LIPIDS

● Serum clear, lipids normal or low. × Serum clear or slightly lactescent, lipids elevated. ○ Serum lactescent, lipids greatly elevated.

In Table I are listed the results of lipid, electrolyte, and gravimetric water determinations made on this and other lactescent sera or chyle, and on the clear subnatant fluids after centrifugation.[4] The discrepancy between the two sets of determinations was greatest in those sera having the highest lipids. While centrifugation removed most of the excess lipid, the concentration of soluble lipids in the subnatant layer was still greater than that of normal serum. This probably accounts for the finding of 91 per cent water in these subnatant layers in contrast to normal serum which contains about 93 per cent water (10).

B. Serum water determinations:

The serum water values estimated by the three methods listed above are given in Table II, together with the lipids, proteins, sodium, and specific gravity. The sodium is given both as concentration in serum and as concentration in serum water.

The serum water determined osmometrically agreed within 1 gm. per cent with that determined gravimetrically, covering a range of serum water from 78 to 99 gm. per cent. Duplicate analyses with the osmometer agreed within 0.5 ml. per cent. It should be pointed out that the gravimetric method would be expected to give results about 0.3 per cent lower than the osmometric method because the first is expressed as weight and the second as volume. The osmometric method was not influenced by hyperlipemia, icterus, or the presence of sugar or nonprotein nitrogen in excessive concentrations. Because of the congealing of lipids during freezing of extremely lactescent sera, the readings in such cases were facilitated if the serum was first diluted by an equal volume of the standard solution.

The relationship between serum water and protein concentration is shown in Figure 1. The anticipated linear relationship (8) was found when the lipids were normal, but when the fatty acids increased much above 30 mEq. per L. the relationship no longer held. The water estimated from the protein concentration then gave falsely high values (Table II) because this method does not take into account the variable contribution of lipids to total solids of serum.

The lipids may influence another commonly used relationship also based on the assumption that proteins are the only important solid of serum. It is seen in Figure 2 that the usually close relationship between serum protein and specific gravity determined gravimetrically does not hold for greatly lactescent sera.

C. Electrolyte studies:

The concentration of sodium in serum and in gravimetrically determined serum water is plotted

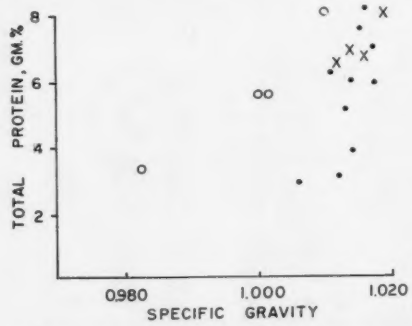

FIG. 2. RELATIONSHIP BETWEEN TOTAL PROTEINS AND SPECIFIC GRAVITY OF SERUM

● Serum clear, lipids normal or low. × Serum clear or slightly lactescent, lipids elevated. ○ Serum lactescent, lipids greatly elevated.

[4] The results of lipid analyses of these sera have been reported in detail (7).

against total fatty acids in Figure 3. If water constitutes 93 per cent of normal serum (10) and if the normal range of serum sodium is 131 to 144 mEq. per L., the normal range of sodium in serum water is 141 to 155 mEq. per L. As in the ultracentrifuge studies, the discrepancy became greater as lipids increased and some very low values of sodium in terms of serum were normal in terms of serum water (Figure 3 and Table II). One value (Yan, Table II) was normal in terms of serum but high in terms of serum water. This serves as a further indication of the displacement of serum water and its solutes by the lipids in lactescent states. A similar "volume effect" of lipids was noted by Popják and McCarthy in lipemic rabbit serum (11), although they did not encounter the extreme hyperlipemia found in some of the subjects of the present study.

An estimate of the water displaced by lipids of lactescent sera would be useful in interpreting electrolyte values in sera in which the concentration of lipids, but not of water, is known. Since protein is the only other solid which displaces water to a major extent, an estimation of this displacement was made by plotting the total fatty acids against the difference between the true serum water determined gravimetrically and that calculated from the protein. It may be seen in Figure 4 that the error in the protein method and hence the displacement of water by lipids increased linearly with increasing concentrations of fatty acid. The magnitude of the error in gm. of water per 100 ml. serum can be estimated roughly by multiplying the total fatty acids in mEq. per L. by the factor 0.04. In effect, this error represents the grams of water displaced by lipids in every 100 ml. of serum. Provided the total fatty acids are known, the degree to which water-soluble substances are displaced by lipids can be estimated. If total lipid weight is known, this estimation can of course be made more directly. While hyperlipemia sufficiently great to cause large displacement of serum water occurs only in greatly lactescent sera, the lipids in clear or only slightly lactescent sera can be high enough to displace as much as 3 gm. per cent.

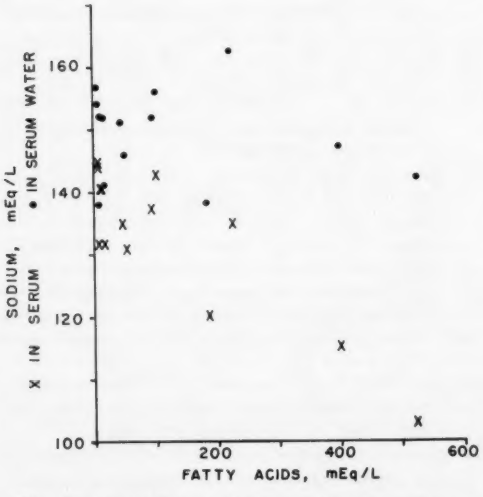

FIG. 3. RELATIONSHIP BETWEEN CONCENTRATION OF SODIUM IN SERUM AND IN SERUM WATER AND CONCENTRATION OF TOTAL SERUM FATTY ACIDS

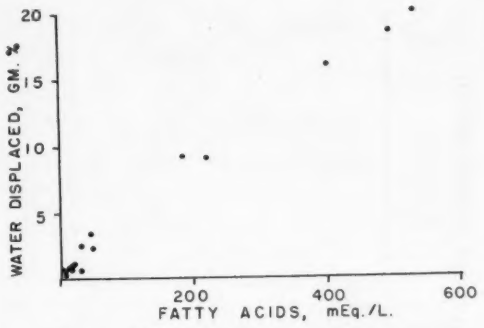

FIG. 4. WATER DISPLACED BY SERUM LIPIDS

The serum lipids are expressed as total fatty acids released by hydrolysis of the lipids. The water displaced is the difference between serum water estimated from protein concentration and that measured gravimetrically.

This method of estimating the water displaced by serum lipids is only approximate, and likely to be of use only in retrospect. With an accurate rapid method for serum water estimation it is preferable to establish the precise concentration of electrolytes in serum water.

In a sense the osmotic pressure itself should give a measure of sodium in terms of its concentration in water, even in very lactescent sera, since the increased lipids in lactescent sera occur chiefly as a separate phase (7), and consequently can not influence the osmotic pressure. The latter conclusion was also reached by Popják and McCarthy (11). However, the true significance

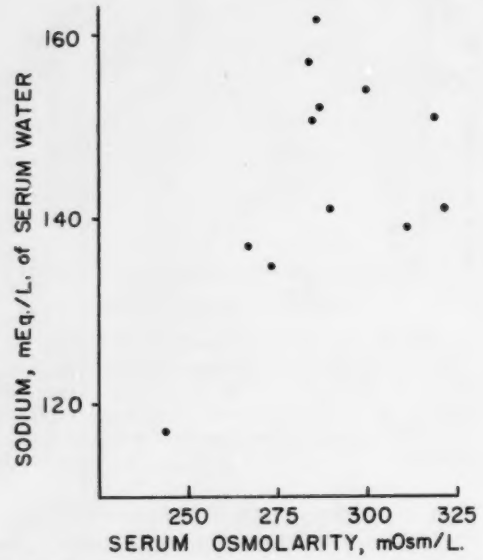

FIG. 5. RELATIONSHIP BETWEEN SERUM OSMOLARITY AND SERUM SODIUM EXPRESSED IN TERMS OF ITS CONCENTRATION IN SERUM WATER

of the osmolarity, shown in Table II, can not be ascertained without knowing the concentration of the solutes other than sodium chloride which influence it. In Figure 5, where the osmolarity is plotted against the concentration of sodium in serum water, it is obvious that the relationship is not a close one in pathological sera.

The fact that the osmometric method for determining serum water worked as well when the serum protein was 3 as it did when it was 8 gm. per cent suggests that the added sodium chloride was evenly dissolved in all the water of serum.

It is clear that for the accurate interpretation of serum sodium concentration, whether it be with regard to hypo- or hypernatremic states, the sodium must be expressed in terms of serum water. When the serum is markedly lactescent this becomes of added importance, the more so because abnormal sodium concentrations are often found in diabetic acidosis and nephrosis, diseases frequently associated with lactescence.

SUMMARY

1. Misleadingly low concentrations of electrolytes were found in the sera of patients with markedly lactescent sera. When the insoluble lipids were removed by ultracentrifugation, the concentrations of electrolytes in the clear subnatant fluids were normal or nearly so.

2. A new method for the simple, rapid determination of serum water is described. It is based on the depression of freezing point caused by adding a known amount of dry sodium chloride to serum. A freezing-point osmometer is employed for these determinations. This method checks within 1 gm. per cent with the gravimetric method.

3. The water in gm. per cent displaced by serum lipids can be roughly estimated as 0.04 time the total fatty acids in mEq. per L.

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HEMATOGENOUS PYELONEPHRITIS IN RATS. I. ITS PATHOGENESIS WHEN PRODUCED BY A SIMPLE NEW METHOD[1,2]

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The study of experimental pyelonephritis has been generally restricted to that form of the disease which develops after ligation of the ureter (1–3). When pyelonephritis is complicated by ureteral ligation, however, a clear distinction cannot be made between the effects of urinary obstruction and those of infection. We have attempted, therefore, to establish experimental pyelonephritis in the absence of hydronephrosis and have succeeded by subjecting rats to a simple new technique requiring only two steps: 1) Injection of bacteria into the blood and 2) Massage of the kidneys through the intact abdominal wall. This method has provided an experimental model which appears to reproduce closely that form of human pyelonephritis occurring in patients with no manifest hydronephrosis. In addition, it dispenses with the cumbersome surgical procedures previously required for the production of experimental pyelonephritis.

This report describes the pathogenesis of pyelonephritis in rats when renal massage is combined with the inoculation of strains of *Escherichia coli* isolated from human infections.

METHODS

I. *The effect of renal massage on the incidence of pyelonephritis in rats inoculated with E. coli*

Sprague-Dawley rats weighing 150 to 200 gm. were divided into two groups of 15 each, and inoculated intracardially with 0.5 ml. of an 18-hour tryptose broth culture of *E. coli*, or approximately 10^8 living bacterial cells as determined by plate counts. This strain of *E. coli* had been isolated from the blood of a patient a few days before the experiment and had been subcultured only twice. Immediately after the bacterial inoculation, one group of 15 rats were subjected to bilateral renal massage. The kidneys of each rat were grasped by thumb and forefinger through the intact abdominal wall (Figure 1) and gently but firmly massaged during ether anesthesia for exactly 5 minutes. In order to maintain a uniform degree of pressure on the massaged kidneys, each period of 5 minutes was divided between two of us instead of confining the total period of massage for each animal to one of several persons. The 15 rats in the second group were anesthetized under ether for 5 minutes but their kidneys were not massaged. The kidneys of a third group were massaged for 5 minutes under ether anesthesia but the rats were not inoculated with bacteria. This third group also served as controls for the experiments in section 3b and are described there.

Within 48 hours, 4 rats in each group had died after the inoculation of *E. coli*. The survivors recovered fully and appeared in excellent condition. At the end of two weeks, the rats were sacrificed by exposure to ether and exsanguination. Approximately 1.0 ml. of blood was divided between trypticase soy broth and blood agar for culture and 0.5 ml. was used for determining urea nitrogen. The kidneys were examined grossly and then each divided into several representative parts for culture and microscopic examination. For culture, the kidney was ground to a pulp with sterile sand, suspended in water, and all of the remaining tissue cultured on blood agar, EMB agar, and tryptose broth. The kidneys were fixed in 10 per cent formalin and the sections stained with hematoxylin and eosin and with Schiff's periodic acid stain.

The procedures described in this experiment for renal massage, bacterial inoculation, and postmortem examination of tissues was that used in all experiments which follow, unless otherwise stated.

II. *Effect of massage on localization of E. coli in kidney as measured by bacterial counts*

From a group of 9 rats inoculated intracardially with *E. coli* (same strain as in I), 3 each were sacrificed at 4 minutes, 1 hour, and 2 hours after unilateral renal massage had been completed. The massage was applied to the right kidney of 4 rats and to the left kidney of the 5 other rats. Immediately before sacrifice by excessive exposure to ether anesthesia, blood was drawn from the heart for plate counts. Each kidney was weighed, ground to a pulp, and suspended in 9.0 ml. of sterile water. Bacterial plate counts were then made on each aqueous suspension of kidney tissue. The entire procedure was carried out aseptically.

[1] Aided by research grant from Public Health Service, H-1955.

[2] Presented in part at the 27th Annual Meeting of The Central Society for Clinical Research, October 30, 1954.

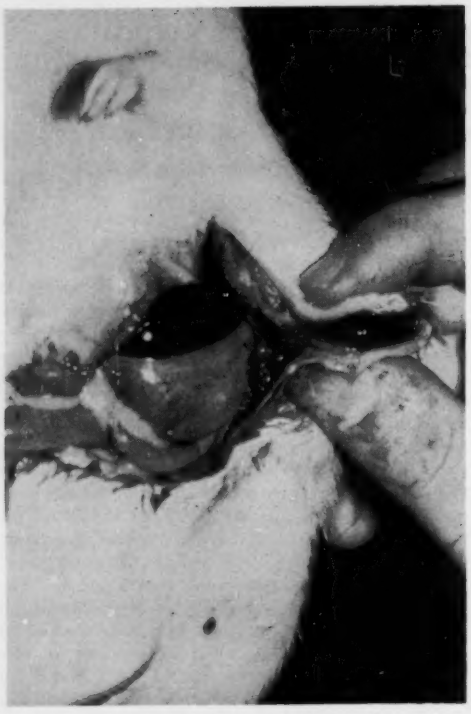


FIG. 1. TECHNIQUE FOR RENAL MASSAGE ILLUSTRATED IN A SACRIFICED RAT

An incision was made to allow visualization of the kidney as it is grasped between the thumb and forefinger.

An additional procedure was carried out with the kidneys of one rat in each group of 3 sacrificed at the specified time intervals. Before suspensions of ground tissue were prepared for plate counts, these kidneys were divided in half and the cortex and medulla from one half were separated. Then each separated portion (intact half, cortex, medulla) of the six kidneys was weighed, ground, and suspended in water.

III. *Serial studies of pathologic changes in massaged kidneys after inoculation of E. coli*

a. *Changes produced by a strain of high virulence.* From a group of 22 rats, 5 each were sacrificed at 2, 24, and 48 hours and the remaining 7 at 7 days after inoculation of approximately 10^8 *E. coli* and bilateral renal massage. Each rat was inoculated with either of two strains of *E. coli* which had been isolated from the urine of patients with pyelonephritis. The two strains appeared to be of equal pathogenicity. Cultures were made of the urine, heart blood, and kidneys; the urinary sediment and the kidneys were examined microscopically; and urea nitrogen was measured on samples of heart blood drawn immediately before sacrifice.

b. *Changes produced by a strain of low virulence.* Rats were divided into 5 groups of 10 each. One group of 10 received bilateral and another group unilateral (right kidney only) renal massage immediately after each animal had been inoculated intracardially with approximately 10^8 *E. coli.* This strain had been isolated from the urine of a patient with pyelonephritis and its virulence was reduced by repeated subculture. Of the three remaining groups of 10 each which served as uninfected controls, one group received bilateral renal massage, a second unilateral renal massage, and the third no massage. Two rats from each of the five groups were sacrificed at 1, 11, 17, 24, and 42 days after inoculation and the same examinations were carried out as in IIIa. In addition, blood pressures were measured just before sacrifice by the tail-plethysmographic method of Williams, Harrison, and Grollman (4).

IV. *Serial studies on urine obtained from living rats by cystotomy*

The experiment in IIIb was repeated but instead of sacrificing the animals, urine was obtained from them by cystotomy during anesthesia. The urines from 2 or more rats in each group were obtained at 2, 3, 5, 6, 8, 10 and 15 weeks after inoculation and then cultured and examined microscopically; but no rat was subjected to cystotomy more than once for the first 6 weeks. After 6 weeks, each of the animals examined was subjected to its second cystotomy. In addition, each of the inoculated rats had received two additional intracardiac injections of the same strain of *E. coli* as follows.

7 weeks—Intracardiac injection of 10^3 *E. coli* with no renal massage. This was done to determine whether the initial renal infection had increased the vulnerability of the kidneys so that pyelonephritis could now be established with relatively small numbers of *E. coli.*

9 weeks—Intracardiac injection of 10^8 *E. coli* accompanied by a second bilateral or unilateral renal massage. This was done to determine whether prior renal infection had rendered the kidneys relatively immune to reinfection.

Cystotomies were done aseptically on rats anesthetized with ether. The bladder was exposed by a small suprapubic incision and urine withdrawn by syringe through a 26 gauge needle. The abdominal wall was repaired with a few silk sutures. At the time of second cystotomies, complete healing of the original cystotomy was usually found.

Before each cystotomy, cardiac blood was drawn from each anesthetized rat for culture and urea nitrogen determination. Blood pressures were measured every other week.

RESULTS

I. *The effect of renal massage on the incidence of pyelonephritis in rats inoculated with E. coli*

The results summarized in Table I disclose that 95 per cent of the massaged kidneys were infected in contrast to only 23 per cent of the control kid-

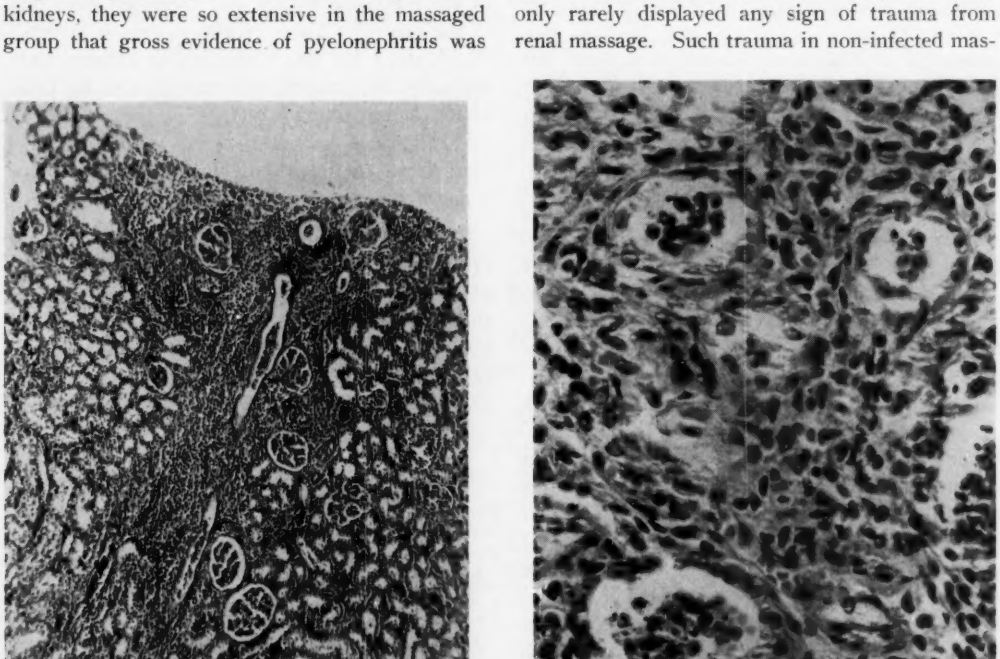
TABLE I*

EFFECT OF RENAL MASSAGE ON THE DEVELOPMENT OF PYELONEPHRITIS AFTER INTRACARDIAC INJECTION OF E. COLI IN RATS

	TOTAL NUMBER OF RATS	E. COLI ISOLATED FROM		GROSS PATHOLOGIC CHANGES	MICROSCOPIC EVIDENCE OF PYELONEPHRITIS
		BLOOD	KIDNEY		
NO MASSAGE	11	0	5	0	0
BOTH KIDNEYS MASSAGED	11	0	21	14	20

* Four rats in each group of 15 died within 48 hours after inoculation of *E. coli*.

neys after inoculating rats with *E. coli*. Even more striking was the observation that although no pathologic changes were found in any control kidneys, they were so extensive in the massaged group that gross evidence of pyelonephritis was found in 64 per cent and microscopic evidence in 91 per cent. Massaged kidneys of rats receiving no inoculation of *E. coli* were invariably sterile and only rarely displayed any sign of trauma from renal massage. Such trauma in non-infected mas-



A B

FIG. 2. MICROSCOPIC CHANGES IN PYELONEPHRITIC KIDNEY INFECTED WITH VIRULENT STRAIN OF *E. Coli* AND SACRIFICED AT 2 WEEKS

A. WEDGE SHAPED AREA OF INTERSTITIAL INFLAMMATION EXTENDING FROM CORTEX TO MEDULLA (× 100)

B. DILATED TUBULES ARE FILLED WITH POLYMORPHONUCLEAR CELLS, EVEN THOUGH CELLS COMPRISING THE INFLAMMATORY EXUDATE IN THE INTERSTITIAL TISSUES ARE VIRTUALLY ALL MONONUCLEAR (× 700)

Only polymorphonuclears are sufficiently motile to move into the tubules in large numbers.

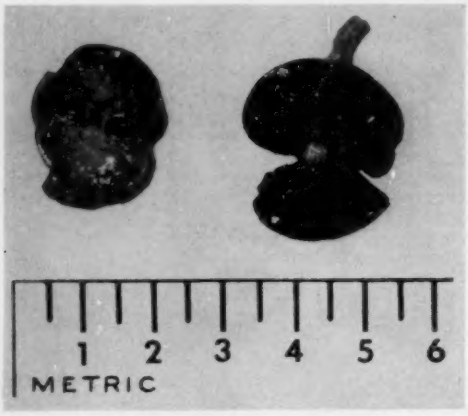


FIG. 3. COMPARISON OF MASSAGED KIDNEY AND CORRESPONDING NON-MASSAGED KIDNEY FROM SAME RAT PHOTOGRAPHED IMMEDIATELY AFTER COMPLETION OF 5-MINUTE PERIOD OF MASSAGE

Note marked hyperemia and enlargement of massaged kidney.

saged kidneys was never manifested by more than a minute hemorrhage or very small areas of tubular necrosis.

The kidneys which developed pyelonephritis were usually enlarged and sometimes showed small abscesses beneath the capsule. On section, there were frequently diffuse areas of suppuration visible. The microscopic appearances of these kidneys were identical to that seen in human pyelonephritis (5). There were wedge shaped areas of interstitial inflammation with its base in the cortex and the apex extending down into the medulla. The process usually spread up to the glomerulus but did not invade it. Although the great majority of cells comprising the inflammatory reaction in the tissues at two weeks were mononuclear, those in the tubules were almost all polymorphonuclear. Many areas containing dilated tubules filled with pus cells were found in association with the interstitial inflammation. The microscopic changes are illustrated in Figure 2.

II. *Effect of massage on localization of E. coli in kidneys as measured by bacterial counts*

Immediately after massage, the kidneys appeared to contain an increased amount of blood and were much heavier than the kidneys on the opposite side which were not massaged (Figure 3). This difference in weight between the massaged and non-massaged kidneys disappeared within two hours as the hyperemic appearance subsided. The number of bacteria per gram of massaged kidney remained about 10 to 100 times greater than that of the control and exhibited a bacterial concentration of 10 to 100 times greater than that of the blood. The higher bacterial count in the massaged kidney resulted from an initial heavy localization of *E. coli* in the cortex alone as illustrated by the 100 fold increase in bacterial count observed in the cortex of massaged over control kidney. Later, the bacterial counts in the medulla of the massaged kidney also increased over that of the control. These results are given in detail in Figure 4 for the 3 rats whose kidneys were separated into cortex and medulla and in Table II for the remaining 6 rats.

III. *Serial studies of pathologic changes in massaged kidneys after inoculation of E. coli*

a. After inoculation of a highly virulent strain of *E. coli* no inflammatory cells appeared in the kidneys or urine during the first 2 hours even though cultures of all kidneys and urines yielded large numbers of *E. coli*. At 24 hours pyuria was observed only in the 2 rats in which the early microscopic signs of pyelonephritis were also found.

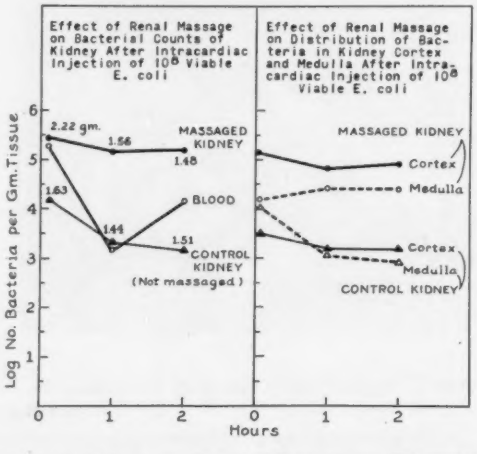


FIG. 4. EFFECT OF RENAL MASSAGE ON BACTERIAL COUNTS OF KIDNEYS AFTER INTRACARDIAC INOCULATION OF *E. Coli*

TABLE II

EFFECT OF RENAL MASSAGE ON BACTERIAL COUNTS OF KIDNEY AFTER INTRACARDIAC INJECTION OF 10^8 VIABLE E. COLI

TIME ELAPSED (MINUTES) AFTER INOC.	IDENTIFICATION NUMBER OF RAT	MASSAGED		TOTAL NO. BACTERIA PER KIDNEY	WEIGHT OF KIDNEY (Gm.)	NO. BACTERIA PER Gm. OF KIDNEY	NO. BACTERIA PER Ml. OF BLOOD
4	1	No	L	85,400	1.0154	84,138	
		Yes	R	740,733	1.2821	577,800	2,330,666
	2	Yes	L	254,866	1.3204	193,080	
		No	R	29,500	1.1294	25,243	2,620,666
60	3	No	L	59,133	1.0081	58,547	
		Yes	R	327,866	1.1782	278,325	15,225
	4	Yes	L	145,400	1.2113	120,165	
		No	R	8,550	1.1695	7,307	11,580
120	5	No	L	35,533	1.1671	30,370	
		Yes	R	449,733	1.2057	371,680	1,173
	6	Yes	L	72,700	1.2619	57,700	
		No	R	18,400	1.2996	14,154	3,013

L = Left Kidney
R = Right Kidney

There were no pus cells in the urine of the other rats examined at 24 hours, and there was no pyelonephritis. Similarly, at 48 hours, the finding of pyuria was limited to those rats whose kidneys displayed the typical gross and microscopic findings of acute pyelonephritis. Yet the urine culture from all rats yielded *E. coli* during the first and second days. By one week, pyelonephritis was present in 13 kidneys of all 7 rats and pyuria was present in the urine of all rats. The results are summarized in Table III.

The earliest evidence of pyelonephritis was found at 24 hours when neutrophils began to attack bacterial masses situated between the tubules. By 48 hours, interstitial abscesses composed of dense accumulations of neutrophils, were

TABLE III *

CORRELATION OF CULTURES AND CELLULAR CHANGES IN EXPERIMENTAL PYELONEPHRITIS

	TIME ELAPSED AFTER INTRACARDIAC INJECTION VIRULENT STRAIN 10^8 E. COLI															
	2 hours				1 day				2 days				7 days			
	U	B	RK	LK	U	B	RK	LK	U	B	RK	LK	U	B	RK	LK
Rat 1	+	+++	+++	+++	+	+	+++	+++	+ P+++	+	+++ PN+++	+++ PN+++	+ P+	0	++ PN++	+ PN++
Rat 2	+	+++	+++	+++	+	+	+++	+++	+	+	+++	+++	+ P++	±	++ PN+++	+ PN+++
Rat 3	+	+++	+++	+++	+	+	+++	+++	±	++	+++	+++	++ P+++	0	+++ PN+++	+++ PN+++
Rat 4	+	+++	+++	+++	+ P+	+	+++ PN ±	+++	+ P++	±	+++ PN+++	+++ PN+++	+ P+++	0	+++ PN++	+
Rat 5	+	+++	+++	+++	+ P+	+	+++ PN ±	+++	++ P++	+	+++ PN+++	+++ PN+++	++ P++	0	+++ PN+++	+ PN+++
Rat 6													++ P++	0	+++ PN+++	+++ PN++
Rat 7													+ P+++	0	+++ PN+++	++ PN++

P = Pyuria
PN = Pyelonephritis

* Cultures were made of blood (B), urine (U), right kidney (RK), and left kidney (LK). (P = pyuria; PN = pyelonephritis; O = sterile.)

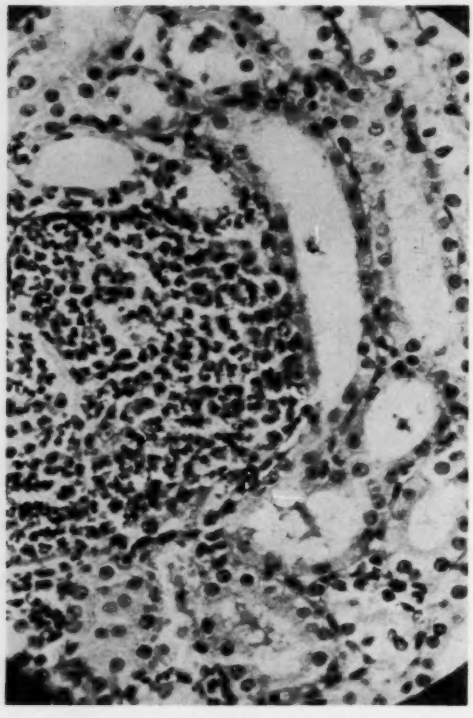


FIG. 5. MICROSCOPIC APPEARANCE OF INTERSTITIAL SUPPURATION IN PYELONEPHRITIC KIDNEY OF RAT SACRIFICED 48 HOURS AFTER INOCULATION OF *E. Coli*

observed grossly and microscopically in the cortex and medulla (Figure 5). At one week, however, many lymphocytes and plasma cells began to participate in the pyelonephritic process. These mononuclears, accompanied by an equal number of neutrophils, comprised a dense infiltrate which usually extended from cortex to medulla in a wedge shaped fashion. The glomeruli were usually spared although the tubules were frequently destroyed or filled with large clumps of neutrophils.

The values for B.U.N. and blood pressure are given in Table IIIA. The B.U.N. ranged from 26 to 46. A few of these values, when compared with those of normal controls given in Table VA, are slightly elevated. The blood pressures on 5 rats taken at 1 week were all normal.

b. After inoculation of a strain of low virulence, the infection is characterized by very few signs of inflammation at any time. The infection was self-limited to about 3 weeks. Despite repeatedly positive cultures of kidney tissue, no renal inflammation was found during the first 17 days and no pyuria developed. In addition, urine cultures were sometimes negative even though the kidneys were infected with *E. coli*. It is particularly noteworthy, however, that microscopic evidence of pyelonephritis sometimes appeared after the infection had disappeared. This late inflammation, which was entirely mononuclear in character, was not accompanied by pyuria. The results are summarized in Table IV. The systolic blood pressure of one animal was 130 mm. Hg at 42 days after bilateral renal massage and inoculation *E. coli*. The blood pressures of all other animals receiving renal massage were 115 or less. The blood pressures in normal control rats were 120 or less. The B.U.N. ranged from 24 to 31 in normals and from 20 to 33 in the animals receiving renal massage with inoculation of *E. coli*. No cultural or pathologic sign of pyelonephritis appeared in any of the control rats.

IV. *Serial studies on urines obtained from living rats by cystotomy*

Table V summarizes the results of serial examinations of urines obtained by cystotomy from rats after renal massage and inoculation of a strain of *E. coli* of low virulence. As in Section IIIb, pyuria was uncommon and renal infection caused by this strain appeared to be self-limited; in these animals it subsided after about 6 weeks. After subsidence of infection, reinoculation with

TABLE IIIA *

TIME ELAPSED AFTER INTRACARDIAC INJECTION 10^8 E. COLI

RAT NUMBER	2 HRS.	1 DAY	2 DAYS	7 DAYS	B.P.
1	44	ND	34	26	ND
2	36	ND	ND	34	ND
3	32	28	30	46	110
4	32	ND	38	32	115
5	34	46	31	42	100
6	34			42	100
7				38	105

Blood urea nitrogens of rats sacrificed serially. Blood pressures just before sacrifice are also given for those examined at 7 days. (ND = No determination)

* These results were obtained on rats listed in Table III.

TABLE IV*

RESULTS OF CULTURE OF URINE (U), BLOOD (B), RIGHT KIDNEY (RK) AND LEFT KIDNEY(LK) UPON SACRIFICE OF RATS

		DAYS ELAPSED AFTER INTRACARDIAC INOCULATION OF 10^8 E. COLI															
		1				11				17				24				42			
		U	B	RK	LK	U	B	RK	LK	U	B	RK	LK	U	B	RK	LK	U	B	RK	LK
MASSAGE BOTH KIDNEYS	Rat 1	0	+	+	+	0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	Rat 2	+	+	+	+	+	0	+	0	0	0	+	0	0	0	0	0 PN	0	0	0 PN	0
MASSAGE RIGHT KIDNEY ONLY	Rat 3	+	+	+	+	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
	Rat 4	0	+	+	+	0	0	+	0	0	0	0	0	0	0	+ PN	0	0	0	0 PN	0

PN = Pyelonephritis
No Pyuria observed in any animal.

* + = isolation of *E. coli*; O = sterile. Isolation of *E. coli* from left kidneys, 1 day after inoculation of rats receiving renal massage only on right side, was attributed to the presence of *E. coli* in the blood contained in those kidneys.

a relatively small number of *E. coli* without renal massage did not lead to recurrent signs of renal infection; but bacteriuria was reestablished at 10 weeks by renal massage in conjunction with the inoculation of larger numbers of *E. coli*. The latter results suggest that upon subsidence of pyelonephritis, the kidneys were neither unduly susceptible to reinfection by small bacterial inoculums, nor immune to reinfection by the usual inoculums. No sign of urinary infection appeared at any time in urines obtained from control rats. The highest blood pressure obtained in any animal was 120 mm. Hg. The B.U.N. in rats subjected to renal massage and inoculation of *E. coli* ranged from 25 to 35, while the B.U.N. of normal controls varied from 26 to 34. These results are given in Table VA.

DISCUSSION

The pathologic appearance of the kidneys of rats, infected with virulent strains of *E. coli*, was almost identical to that seen in human pyelonephritis caused by *E. coli*. In both cases, the inflammation is interstitial and extends in a wedge shaped fashion from its base in the cortex down to the medulla (5). In rats, as in man, it was also found that urinary findings may be normal despite extensive infection in the interstitial tissue of the kidney (6). The acute pyelonephritis in

TABLE V*

RESULTS OF URINE CULTURE FOR E. COLI OBTAINED BY CYSTOTOMY WITHOUT SACRIFICING RATS

		WEEKS ELAPSED AFTER FIRST INTRACARDIAC INJECTION OF 10^8 E. COLI								
		2	3	5	6	7	8	9	10	15
MASSAGE BOTH KIDNEYS	RAT 1	+++	+ P	0	0	Intra cardiac injection 10^3 E. Coli NO MASSAGE	0	Intra cardiac injection 10^6 E. Coli and kidneys massaged	+	0
	RAT 2	+++	+	0	0		0		0	+
MASSAGE RIGHT KIDNEY ONLY	RAT 3	++	0	0	0		0		0	+
	RAT 4	++	0	+	+		0		+ P	0

P = Pyuria
All blood culture sterile.
BUN and Blood pressure normal on all animals.

* The designations "Rat 1, Rat 2, Rat 3, Rat 4" are used only to identify rats within the group examined on a given day; no rat was subjected to cystotomy more than once for the first 6 weeks. In addition to those listed, urines were also sterile from 2 rats receiving bilateral renal massage at 6 weeks and from 2 rats receiving unilateral renal massage at 15 weeks.

TABLE VA*

	Inoc. with E. Coli at 0, 7, & 9 wks.	WEEKS ELAPSED AFTER FIRST INTRACARDIAC INJECTION OF 10^8 E. COLI						
		2	3	5	6	8	10	15
Massage both kidneys	Yes	25 117	29 111	27 112	30 112	26 107	35 109	ND 110
Massage right kidney	Yes	28 116	25 107	30 109	25 112	33 110	ND 107	ND 112
No massage of kidneys	Yes	30 112	31 106	27 106	29 104	30 103	31 106	ND 109
Massage both kidneys	No	29 108	29 109	26 107	31 113	27 109	31 115	ND 115
No massage (normal controls)	No	34 109	29 104	28 106	32 110	ND 109	26 108	ND 107

Blood urea nitrogens and blood pressures of rats examined periodically by cystotomy.

*The B.U.N. is the upper value, and the blood pressure the lower value given at each time interval for each group of rats. The values given are averages for all rats examined in a group. (N.D. = no determination.) These results were obtained on rats listed in Table V and on the controls.

rats and in man also resemble each other in that hypertension and usually uremia are absent in both cases. These similarities between pyelonephritis in humans and in these rats suggest that the experimental disease may be used as a model for studying certain aspects of human pyelonephritis.

Renal massage appears to favor the development of hematogenous pyelonephritis in bacteremic rats by causing increased numbers of bacteria to localize in the kidney. It is not clear from the present data whether this heavy localization of *E. coli* resulted from renal damage, urinary obstruction, or from the delivery of more bacteria through increased renal flow of infected blood. Although no obstruction of the urinary tract and only rare renal damage were evident at postmortem examination of the massaged kidneys, these abnormalities might have been detectable if function studies had been carried out. Likewise, in the absence of hemodynamic studies, it cannot be established whether the hyperemic appearance of the massaged kidneys represents increased blood flow or simple engorgement and stasis. It is also possible that the relatively poor lymphatic supply of the kidney was damaged and not adequate to remove the excessive numbers of bacteria which could then establish infection. Pierce (7) has demonstrated that in contrast to skin, intestine, or uterus, there are very few renal lymphatics present to take over as emergency units to handle any additional load over that normally present. In the present experiments, bacteria which lodged in the kidneys remained there even though they were of such low virulence that they induced no necrosis or inflammatory reaction. This would account for the persistence of *E. coli* in the kidney and urine for several weeks in the absence of inflammatory cells.

Once the experimental infection was established, its manifestations varied with the virulence of the organism. Highly virulent strains led to suppuration and pyuria. Strains of lower virulence produced a mild self-limited infection with sometimes little or no sign of renal inflammation. Upon analysis of these two types of infectious processes in the experimental animals, several points become evident which help explain certain clinical findings in human pyelonephritis which are not well understood. For example, it was found in suppurative experimental pyelonephritis that, although the cellular reaction in the tissues was predominantly mononuclear by two weeks, virtually all the cells in the tubules were polymorphonuclears. This difference is accounted for by the great motility of polymorphonuclears which enables them to move out of the inflamed areas into the tubules. This experimental observation is useful in explaining

why the polymorphonuclear is almost the only leukocyte found in the urinary sediment in human pyelonephritis and why the urinary sediment in chronic pyelonephritis frequently contains no cells after the renal inflammation becomes lymphocytic in character.

During the experimental renal infection by strains of low virulence, there was a different reason for the absence of pyuria. Here the cellular reaction in the kidney itself was so slight, if present at all, that there was often no significant focus of polymorphonuclears to supply the tubules. In such an infection, only motile bacteria were present in the urine and even these were often absent. Whether a comparable form of acute non-suppurative pyelonephritis occurs in human beings can only be conjectured, however, because pathologic and cultural examinations of such kidneys would be almost impossible to secure. Yet there is abundant evidence to suggest that a clinical entity of this type exists. The syndrome of fever, chills, sweats, and bacteriuria occurring in the absence of flank pain, costovertebral angle tenderness, and pyuria is not uncommonly produced by *E. coli*. The course of this disease is generally self-limited to a few days or weeks, but may recur for many years and lead to chronic pyelonephritis (6). In this connection, it is of interest that in the experimental infection, there was not only a lack of immunity against subsequent attacks of *E. coli* pyelonephritis, but also evidence which suggested that the pyelonephritic changes may first appear as the infection vanishes (Table III). Thus it may be possible for repeated infections by the same bacterial species to occur and initiate inflammatory reactions which perpetuate themselves after the bacteria disappear.

These phenomena and others relating to chronic pyelonephritis are under investigation in rats whose renal infections have persisted for over 6 months. It is anticipated that these studies now in progress may illuminate some of the problems related to chronic human pyelonephritis.

CONCLUSIONS

Renal massage appears to favor development of hematogenous pyelonephritis in bacteremic rats by causing large numbers of bacteria to localize in the kidney. The severity of the pyelonephritis depends on both renal massage and bacterial virulence. Renal infection (proved by positive cultures of kidneys) may exist in the absence of pyuria and bacteriuria.

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STUDIES ON COPPER METABOLISM. XIV. COPPER, CERULOPLASMIN AND OXIDASE ACTIVITY IN SERA OF NORMAL HUMAN SUBJECTS, PREGNANT WOMEN, AND PATIENTS WITH INFECTION, HEPATOLENTICULAR DEGENERATION AND THE NEPHROTIC SYNDROME [1]

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Ceruloplasmin, the blue copper-containing protein of serum, was first isolated in 1948 by Holmberg and Laurell (1). They characterized this protein as an alpha 2 globulin with a copper content of approximately 0.32 per cent,[3] a molecular weight of about 151,000 and 8 atoms of copper per molecule. The substance has been shown to exhibit oxidase activity with a variety of substrates. The enzyme is most active with paraphenylene diamine (2, 3).

Holmberg and Laurell (4) observed that ceruloplasmin could be precipitated from normal human serum made 50 per cent saturated with ammonium sulfate. However, approximately 2 to 12 per cent of the total copper remained in the soluble "albumin" fraction. They suggested that the copper remaining in the albumin fraction might be attached to globulin which could not be completely separated from the albumin by ammonium sulfate precipitation. More recently, in our laboratory (5), it was demonstrated that there is a small fraction of the plasma copper (0 to 17 per cent) in normal subjects which reacts directly with the copper colorimetric reagent, sodium diethyldithiocarbamate. This fraction has been referred to as the "direct-reacting fraction" of plasma or serum copper and evidence has been brought forward that it is this fraction which is actively concerned in the transportation of copper. The remainder of the copper in plasma does not react directly with the carbamate reagent and has been referred to as the "indirect-reacting fraction" of plasma copper. This presumably corresponds to ceruloplasmin. Recently, Bearn and Kunkel (6), by the use of zone electrophoresis, have presented evidence that the direct-reacting copper is bound to serum albumin.

It has been demonstrated repeatedly that the total plasma copper level is elevated in the last trimester of pregnancy (7, 8) and in patients with infections (7–9). Holmberg and Laurell (4, 10) demonstrated that in such patients most of the copper is associated with the globulin fraction and that there is a high degree of correlation between the copper content of the sera and the oxidase activity. In pregnant women, the hypercupremia is due mainly to an increase in ceruloplasmin (11); although there is a modest increase in the direct-reacting fraction as well (5).

It is now known that hypocupremia is present in most patients with Wilson's disease (8, 12, 13) and in some patients with the nephrotic syndrome (14). In Wilson's disease it has been demonstrated that there is a marked decrease in the indirect-reacting fraction of plasma copper and an increase in the direct-reacting fraction (13). Scheinberg and Gitlin (15), by the use of specific immunologic techniques, measured ceruloplasmin directly and found it to be markedly reduced. In

[1] This investigation was supported by a research grant (C-2231) from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Public Health Research Fellow of the National Heart Institute.

[3] If ceruloplasmin contains 8 atoms of copper per mole, the correct molecular weight for the observed copper concentration of 0.32 per cent would be 159,000. The exact molecular weight of ceruloplasmin is uncertain. Holmberg and Laurell gave the figure as "approximately" 151,000. However, if this is correct, the copper concentration should be 0.336 per cent. We have arbitrarily chosen 0.32 per cent for the purposes of the calculations in this paper, since this is the value which Holmberg and Laurell actually found.

the nephrotic syndrome (14), it has been suggested that the hypocupremia is the consequence of the loss of ceruloplasmin in the urine. Although patients with the nephrotic syndrome have been found to excrete increased quantities of copper in the urine, it has not been demonstrated conclusively that the copper is excreted as ceruloplasmin.

The purpose of this paper is to present the results of measurements of total serum copper, direct-reacting copper, indirect-reacting copper, ceruloplasmin and oxidase activity in the sera of normal human subjects, pregnant women, and patients with infection, Wilson's disease, the nephrotic syndrome, and miscellaneous conditions. We have also measured the ceruloplasmin concentration in the urine of patients with Wilson's disease and the nephrotic syndrome and in several tissues of one patient with Wilson's disease and of two subjects without Wilson's disease.

METHODS

The patients used in this study, in most instances, were not hospitalized. The pregnant women were all in the third trimester of an uncomplicated pregnancy. Five of the patients (Del. H., Dar. H., D. C., R. K., and J. S.) with Wilson's disease have been reported in a previous publication from this laboratory (13). Two additional cases (J. Si. and B. S.) have been studied in detail in this laboratory since the previous publication. The remainder of the sera from patients with Wilson's disease were kindly sent to us by various physicians.[4] One of these patients (H. C.) has been the subject of two reports (16, 17).

All but three of the patients with the nephrotic syndrome who were studied manifested the classical features of this condition; namely, intractable edema, massive proteinuria, lipemia and hypoalbuminemia. Four (C. E., C. S., H. B., and R. P.) were adults in whom the nephrosis was presumably secondary to chronic glomerulonephritis. Five[5] (W. W., G. H., H. A., L. L., and S. C.) were children. In these the syndrome could be classified as the so-called "pure" or "lipoid nephrosis."

All analyses were performed on serum which had been collected carefully to prevent contamination with copper. Measurements of direct-reacting copper and oxidase activity were performed on fresh serum only.

The methods for the determination of total serum copper (5), the direct-reacting fraction of serum copper (5), urine copper (14), tissue copper (18), and urine protein (14) have been described in previous publications. The oxidase activity of the serum was estimated by the method of Holmberg and Laurell (2) employing paraphenylene diamine as a substrate.

Ceruloplasmin was measured immunologically by the use of rabbit anti-human ceruloplasmin serum which had been absorbed with serum from a patient with Wilson's disease (15). Rabbits were immunized by the Lipton and Freund adjuvant technique (19) with ceruloplasmin isolated in this laboratory (1) from human serum. The sample (C-V) used for immunization contained 0.23 per cent copper and 14.0 per cent nitrogen. Both intracutaneous and intramuscular routes were used simultaneously. Each rabbit received one injection of 4 mg. ceruloplasmin weekly for six weeks and was bled by cardiac puncture seven days after the last injection.

The pooled serum was absorbed twice with 0.5 ml. portions of serum from Del. H. for 48 hours at 0 to 5° C. and was then tested by the Oudin technique (20). A concentrated solution of ceruloplasmin (5.25 mg. per ml.) in 0.15 M saline, normal serum, and serum from a patient with Wilson's disease were used as antigens. Absorptions with 0.5 ml. portions of serum from Del. H. were continued until only one zone was obtained.

The sera were calibrated with human ceruloplasmin. It was assumed that the copper concentration of pure ceruloplasmin is 0.32 per cent. The amount of antibody was estimated by a modification of the colorimetric method of Heidelberger and MacPherson (21, 22). One ml. of a 1:4 dilution of antiserum was chilled to 0° C. and 1.0 to 3.0 ml. of the appropriately diluted test solution was added. The total volume was made up to 4.0 ml. with cold 0.15 M saline and the tubes were kept at 0 to 5° C. for four days and then centrifuged at 0 to 4° C. The precipitate, after washing with 3.0 ml. and then 2.5 ml. of cold saline, was dissolved in three drops of N/10 sodium hydroxide and diluted to 2.5 ml. with water. The color was developed as outlined by Heidelberger and MacPherson. The densities of the solutions were measured in a Beckman Model B Spectrophotometer at a wave length of 650 mμ and the concentration of ceruloplasmin was determined from the calibration curve. The supernatant solutions from the precipitin tests were checked for excess antibody. All determinations were run in the region of antibody excess since the calibration curve for the antiserum is valid only in this range.

Tissues for ceruloplasmin analysis were stored at −30° C. until used. Small samples (1 to 2 gm.) were homogenized in a Virtis homogenizer (Model 6-105) with 0.15 M saline in the cold. The homogenates were centrifuged at 12,000 r.p.m. for 30 minutes. Cerulo-

[4] We are indebted to the following physicians for sending us specimens of serum and for granting us permission to publish the results of the analyses; Dr. R. E. Hodges, University of Iowa, Iowa City, Iowa (patients R. K., W. K., and J. S.); Dr. W. N. Jensen, Veterans Administration Hospital, Durham, N. C. (patient W. S.); Dr. J. G. Palmer, University of North Carolina, Chapel Hill, N. C. (patients N. P. and A. S.); Dr. C. G. Warnock, Royal Victoria Hospital, Belfast, Ireland (patients H. C. and P. C.); Dr. G. S. Ferriss, Charity Hospital of Louisiana, New Orleans, La. (patient F. D.).

[5] We are indebted to Dr. Phillip Sturgeon, Children's Hospital Society, Los Angeles, Cal., for sending us sera from these patients.

TABLE I

Normal human subjects

Subject	Sex	Total serum copper μg/100 ml.	Direct-reacting copper μg/100 ml.	Indirect-reacting copper μg/100 ml.	Ceruloplasmin mg./100 ml.	Oxidase activity μM O_2/ml./hr.
H. M.	M	92	0	92	27	4.3
S. A.	M	107	0	107	30	3.4
A. S.	M	101	0	101	33	3.5
G. C.	M	104	6	98	31	3.0
J. M.	M	114	0	114	38	4.7
L. H.	F	110	11	99	34	3.8
M. Y.	F	116	6	110	38	4.8
G. T.	F	111	17	94	38	4.1
A. W.	F	123	0	123	38	4.7
N. B.	F	97	9	88	30	3.0
Mean		108	5	103	34	3.9
±S.D.		±9	±6	±11	±4	±0.7
Range		92–123	0–17	88–114	27–38	3.0–4.7

plasmin was determined on aliquots of the supernatant solutions as outlined above. Tissue extracts were set up against normal rabbit serum to test for non-specific precipitation. These tests were negative. In order to correct for the ceruloplasmin contributed by the serum trapped in the tissues of the two control subjects, a hemoglobin determination was done on aliquots of the supernatant solutions.

RESULTS

Normal human subjects

The data for 10 normal subjects are presented in Table I. The mean value for total serum copper of 108 μg/per 100 ml. corresponds well with the normal mean determined previously in this laboratory in a much larger group (14). Likewise, the mean value for the direct-reacting fraction of 5 μg per 100 ml. corresponds well with the mean values of 4 and 8 μg per 100 ml., respectively, previously reported (5, 13).

The mean value for ceruloplasmin concentration was 34 mg. per 100 ml. and for oxidase activity, 3.9 μM O_2 per ml. per hr.

Assuming that ceruloplasmin contains 0.32 per cent copper (1), the amount of copper accounted for in the ceruloplasmin is 109 μg per 100 ml. This figure corresponds well with the mean determined value for total serum copper but is about 6 per cent above the mean value for the indirect-reacting fraction.

Pregnancy

Studies were made in 10 women. The results are presented in Table II. The total serum cop-

TABLE II

Pregnancy
(Third trimester)

Patient	Total serum copper μg/100 ml.	Direct-reacting copper μg/100 ml.	Indirect-reacting copper μg/100 ml.	Ceruloplasmin mg./100 ml.	Oxidase activity μM O_2/ml./hr.
F. K.	267	51	216	86	10.1
F. F.	222	31	191	68	10.7
A. G.	297	20	277	107	12.6
E. K.	249	17	232	83	11.7
S. L.	273	26	247	92	9.3
W. L.	216	37	179	62	6.6
K. W.	223	11	212	80	8.2
J. S.	216	20	196	68	6.1
S. B.	288	29	259	94	9.1
R. M.	324	49	275	99	11.5
Mean	257	29	228	84	9.6
±S.D.	±38	±13	±35	±15	±2.2
Range	216–297	11–51	179–277	62–107	6.6–12.6

TABLE III

Infection

Patient	Disease	Total serum copper μg/100 ml.	Direct-reacting copper μg/100 ml.	Indirect-reacting copper μg/100 ml.	Ceruloplasmin mg./100 ml.	Oxidase activity μM O_2/ml./hr.
R. P.	Tuberculosis	178	26	152	63	8.1
E. C.	Tuberculosis	172	11	161	60	8.7
W. H.	Tuberculosis	154	6	148	60	7.2
E. W.	Pneumonia	200	23	177	72	7.7
I. H.	Pneumonia	219	17	202	75	9.3
E. M.	Staph. abscess*	219	26	193	83	8.0
B. K.	Sinusitis	203	11	192	61	7.6
Mean		192	17	175	68	8.1
Range		154–219	6–26	148–202	60–83	7.2–9.3

* Complicating acute leukemia.

per, direct-reacting serum copper, ceruloplasmin and oxidase activity were all greatly increased above normal. The mean total serum copper was increased 138 per cent, the mean direct-reacting 480 per cent, the mean ceruloplasmin 147 per cent, and the mean oxidase activity 146 per cent above the normal mean values. The amount of copper accounted for in ceruloplasmin, on a basis of 0.32 per cent copper, was 269 μg per 100 ml. This value is 4.7 per cent greater than the determined total serum copper and greater than the mean indirect-reacting copper by 18 per cent.

Infection

The values observed in seven patients with infection (Table III) were similar to those in pregnancy although the changes were not as great. The mean total serum copper was increased 78 per cent above the normal mean, the direct-reacting copper 240 per cent, the ceruloplasmin 100 per cent, and the oxidase activity 108 per cent. The amount of copper accounted for by ceruloplasmin was 218 μg per cent. Again, this was greater (12 per cent) than the determined total serum copper and indirect-reacting copper (24 per cent). In the case of E. M., the copper present in ceruloplasmin by calculation was 22 per cent greater than the determined total plasma copper and 39 per cent greater than the indirect-reacting value. It is of interest that this patient had hyperglobulinemia with 5.6 grams of total globulin per 100 ml. of serum.

Nephrotic syndrome

The results in seven patients with the nephrotic syndrome are presented in Table IV. In only four of the nine patients was hypocupremia present. The mean value for total serum copper for the group was 86 μg per 100 ml. The ceruloplasmin level was increased above the upper limit of normal in two patients (C. E. and H. B.), was within the normal range in two patients (G. H. and R. P.), and was below the lower limit of normal in the five remaining patients. It is of

TABLE IV

Nephrotic syndrome

Patient	Total serum copper μg/100 ml.	Direct-reacting copper μg/100 ml.	Indirect-reacting copper μg/100 ml.	Ceruloplasmin mg./100 ml.	Oxidase activity μM O_2/ml./hr.
C. E.	129	8	121	54	6.2
H. B.	126	17	109	46	4.6
G. H.	102			29	
R. P.	96	26	70	29	2.5
L. L.	95			26	
C. S.	72	26	46	21	2.3
S. C.	67			12	
W. W.	48			13	
H. A.	36			12	

TABLE V

Protein, copper, and ceruloplasmin in the urine of four patients with the nephrotic syndrome

Patient	Protein gm./24 hrs.	Copper μg/24 hrs.	Ceruloplasmin mg./24 hrs.
Normal	<0.8	0–26	0
R. P.	4	205	50
C. E.	5	239	55
C. S.	11	246	46
H. B.	18	410	75

interest that in three of the patients (S. C., W. W., and H. A.) the ceruloplasmin values were as low as those seen in hepatolenticular degeneration. Direct-reacting copper was measured in only four patients. In two of these (R. P. and C. S.) the values were above the upper limit of normal.

From Table IV it is apparent that there was high degree of correlation between the total serum copper level and the ceruloplasmin and oxidase activity.

The excretion of protein, copper, and ceruloplasmin in the urine was measured in four patients (Table V). From 46 to 75 mg. of ceruloplasmin were excreted in the urine per 24 hours.[6]

Hepatolenticular degeneration

The results in 14 patients with hepatolenticular degeneration are shown in Table VI. The serum copper level was less than 68 μg per cent, the lower limit of normal (23), in 11 of the 14 patients. In the remaining three patients (D. C., A. S., and P. C.) the serum copper levels were 86, 100, and 77 μg per 100 ml., respectively. In all of the patients the ceruloplasmin concentration was less than the lower limit of normal (Table I). The direct-reacting copper was increased above normal in 4 of the 5 patients on whom this was measured. In all five of the patients studied, the indirect-reacting fraction was reduced below normal. The oxidase activity was greatly reduced in all four of the patients on whom this determination was performed.

The ceruloplasmin concentration in the different patients ranged between 2 and 19 mg. per 100 ml. Between the range of 2 to 11 mg. per 100 ml. of ceruloplasmin, there was no correlation with the total serum copper level which ranged from 35 to 60 μg per 100 ml. In the three patients with ceruloplasmin levels greater than 11 mg. per 100 ml., the serum copper levels were greater (77, 100, and 86 μg per 100 ml.) than in those with the lower ceruloplasmin levels. There was good correlation between the indirect-reacting copper and the ceruloplasmin level in the few patients studied.

The clinical data on the patients with Wilson's disease are summarized briefly in Table VII. As is evident in this table and in Table VI, there was no obvious correlation between the ceruloplasmin concentration in the serum and the duration or

[6] Oxidase activity was demonstrable in the urine of these patients and was not detectable in normal urine.

TABLE VI

Hepatolenticular degeneration

Patient	Total serum copper μg/100 ml.	Direct-reacting copper μg/100 ml.	Indirect-reacting copper μg/100 ml.	Ceruloplasmin mg./100 ml.	Oxidase activity μM O_2/ml./hr.
B. S.	57	50	7	2	0.0
F. D.	43			4	
J. Si.	50	37	13	4	0.0
W. K.	52			6	
W. S.	52			8	
Dar. H.	53	34	19	8	0.2
Del. H.	35	11	24	9	
H. C.	46			9	
J. S.	53			10	
R. K.	60			10	
N. P.	60			11	
P. C.	77			15	
A. S.	100			17	
D. C.	86	26	60	19	2.1
Mean	59	32	25	9	0.6

TABLE VII

Clinical data on cases of hepatolenticular degeneration

Patient	Age *years*	Sex	Duration symptoms *years*	K-F rings	B.S.P. retention* %	Neurologic manifestations			Hyper-cupriuria	Hyper-amino-aciduria
						Dysarthria	Tremor	Spasticity		
B. S.	18	M	2	+	5	+	+	0	+	+
F. D.	23	M	4	+	11	+	+	0	+	
J. Si.	35	M	12	+	10	+	+	+	+	+
W. K.	31	M	5	+	5	+	+	0	+	+
W. S.	29	M	3	+	5	+	+	0	+	
Dar. H.	11	F	0	+	26	0	0	0	+	0
Del. H.	15	M	6	+	28	+	+	+	+	+
H. C.	17	M	6	+		0	0	0	+	+
J. S.	30	F	4	+	2	+	+	+	+	+
R. K.	22	M	13	+	8	+	+	+	+	+
N. P.	31	F	0	+	13	0	+	0	+	
P. C.	14	M		0		+	+	0	+	0
A. S.	19	M	0	+	20	0	0	0	+	
D. C.	29	F	4	+	32	+	+	+	+	+

* Forty-five minutes following the injection of 5 mg. per Kg.

severity of the clinical manifestations in this very small series of patients. Patient D. C. with the highest ceruloplasmin concentration has been observed to have clinical signs of the disease for at least four years. Her liver function is abnormal and there is well advanced neurologic disease (13). Patient B. S. with the lowest ceruloplasmin concentration has shown clinical signs for only two years, all of the liver function tests are essentially normal, and he has only a moderate degree of dysarthria and minimal tremor of the left arm. Patient Del. H. with a ceruloplasmin level of 9 mg. per 100 ml. died with severe liver impairment and neurologic disease one month after the ceruloplasmin determination (13). It may be added that there was no correlation between the concentration of ceruloplasmin and the age of the patient, the duration of clinical symptoms or signs, the presence or absence of neurologic disease, or the degree of liver function impairment as determined by bromsulfalein retention.

The urine of three of the patients (D. C., Dar. H., and F. D.) was examined for ceruloplasmin by the immunologic technique and none or, at most, traces were found even though all three of the patients were excreting abnormally large quantities of copper in the urine.

Neurologic disorders other than hepatolenticular degeneration

In order to learn whether or not a decrease in ceruloplasmin occurs in neurologic conditions other than Wilson's disease, determinations were performed on four patients with the Parkinsonian syndrome, three patients with schizophrenia, two patients with multiple sclerosis, one with a familial tremor, one with myasthenia gravis, and two with Huntington's chorea. The results are presented in Table VIII.

In none of the patients studied was there a significant decrease below the normal in total serum copper, indirect-reacting copper, or ceruloplasmin. In one patient with schizophrenia (D. K.) there was an increase in the direct-reacting fraction and a slightly low indirect-reacting copper. The oxidase activity was distinctly reduced. In two other patients with neurologic disease (J. P. and W. B.) the oxidase activity was reduced even though the ceruloplasmin levels were normal. In the patient with myasthenia gravis (M. H.) the ceruloplasmin level was at the lower limits of normal. We do not regard these slight deviations as being of any significance.

Ceruloplasmin in tissues

The concentration of ceruloplasmin was measured in several tissues of one patient (Del. H.) with hepatolenticular degeneration and one control subject (K. D., traumatic death). The concentration in the kidney was measured in one additional control subject (H. C., diabetes mellitus). The results are presented in Table IX.

Small quantities of ceruloplasmin were detected in the liver and kidney of the control subjects, and none was detected in the spleen, basal ganglion, cerebellum, bile, or erythrocytes. Even in the

TABLE VIII

Neurologic disorders other than Wilson's disease

Patient	Condition	Total serum copper μg/100 ml.	Direct-reacting copper μg/100 ml.	Indirect-reacting copper μg/100 ml.	Ceruloplasmin mg./100 ml.	Oxidase activity μM O_2/ml./hr.
A. L.	Parkinsonian syndrome	174	3	171	75	6.4
M. R.	Parkinsonian syndrome	144	0	144	51	5.7
J. Z.	Parkinsonian syndrome	169	20	149	59	6.3
J. P.	Parkinsonian syndrome	104	9	95	33	2.0
E. C.	Multiple sclerosis	129	0	129	59	5.3
J. H.	Multiple sclerosis	156	9	147	78	6.9
R. P.	Schizophrenia	150	9	141	58	6.0
K. K.	Schizophrenia	123	9	114	38	4.2
D. K.	Schizophrenia	93	26	67	32	1.5
W. B.	Familial tremor	107	6	101	31	2.5
M. H.	Myasthenia gravis	125	0	125	26	4.7
T. T.	Huntington's chorea	141			31	
A. D.	Huntington's chorea	111			28	

liver and kidney the copper in ceruloplasmin accounted for only a small fraction of the total copper present. In the liver and kidney of the patient with Wilson's disease the total copper was increased as compared with that found in the "controls" and almost none of this was in the form of ceruloplasmin.

DISCUSSION

The data presented demonstrate a high degree of correlation between the total plasma copper level and the concentration of ceruloplasmin (Figure 1), between the indirect-reacting fraction of plasma copper and ceruloplasmin (Figure 2), and between the serum oxidase activity and ceruloplasmin concentration (Figure 3) in normal human subjects, in pregnant women and in patients with infections, the nephrotic syndrome and certain neurologic disorders other than Wilson's disease. The mean values for ceruloplasmin in normal subjects and in patients with Wilson's disease are in excellent agreement with those obtained by Scheinberg and Gitlin (15). However, the mean values of 84 mg. per 100 ml. for ceruloplasmin and 29 μg per 100 ml. for the direct-reacting copper in pregnant women in the last tri-

TABLE IX

Ceruloplasmin in tissues

Tissue	Condition	Subject	Copper μg/gm.*	Ceruloplasmin μg/gm.*	Proportion of total copper in ceruloplasmin %
Liver μg/gm.	Control	K. D.	3.9	55	4.5
	W. D.†	Del. H.	98.4	20	0.1
Spleen μg/gm.	Control	K. D.	0.7	0	0
	W. D.	Del. H.	1.5	0	0
Kidney μg/gm.	Control	K. D.	1.9	73	12.1
	Control	H. C.	4.1	50	3.9
	W. D.	Del. H.	8.7	12	0.4
Basal ganglion μg/gm.	Control	K. D.	6.0	0	0
	W. D.	Del. H.	56.4	0	0
Cerebellum μg/gm.	W. D.	Del. H.	67.4	0	0
Bile μg/100 ml.	W. D.	Del. H.	19.0	0	0
Erythrocytes μg/100 ml.	Control	H. M.	115.0	0	0

* Wet tissue.
† W. D. refers to Wilson's Disease.

mester are higher than the mean values of 56 mg. and 10.6 μg found by Scheinberg, Cook, and Murphy (11) in women at the time of delivery.

In general, the values for ceruloplasmin copper, calculated on the basis of 0.32 per cent copper in ceruloplasmin, were higher than the corresponding indirect-copper values. In some instances, these were even higher than the total copper values. This discrepancy was greater in sera with high copper concentrations than in sera with low or normal concentrations of copper. In patient E. M. (Table III) with hyperglobulinemia, the

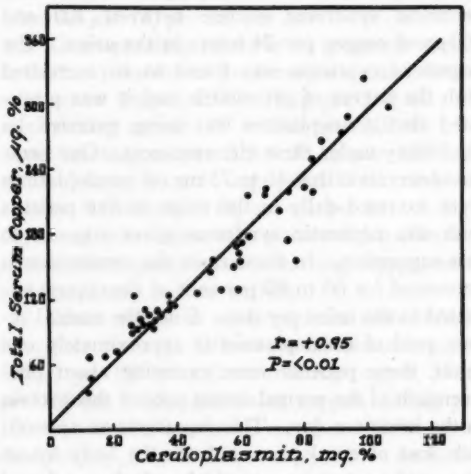

FIG. 1. THE CORRELATION BETWEEN THE TOTAL SERUM COPPER AND CERULOPLASMIN

All of the values for normal subjects, pregnant women, and patients with infection, the nephrotic syndrome and neurologic disease other than hepatolenticular degeneration have been plotted. The diagonal line was plotted by the method of least squares. r refers to the correlation coefficient. P refers to the probability of such a correlation occurring by chance.

calculated ceruloplasmin copper was 22 per cent greater than the total serum copper. These results suggest that the antiserum used contained antibodies to proteins other than ceruloplasmin. In order to test this possibility the serum of A. L. (Table VIII) with a total copper of 174 μg per 100 ml. and a calculated ceruloplasmin copper of 240 μg per 100 ml. was studied by the Oudin agar diffusion technique. Only a single zone was found. Although this does not entirely eliminate the possibility that antibodies to proteins other

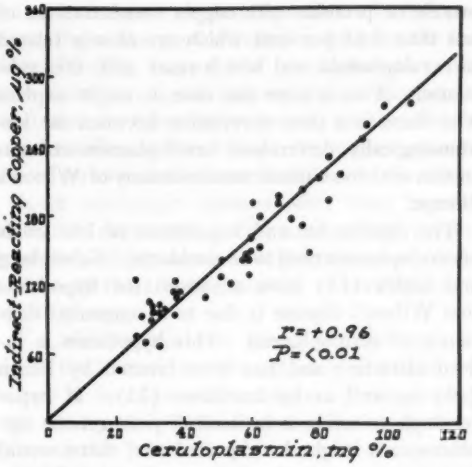

FIG. 2. THE CORRELATION BETWEEN THE INDIRECT-REACTING FRACTION OF SERUM COPPER AND CERULOPLASMIN

For explanation see legend for Figure 1.

than ceruloplasmin were present, it makes this explanation less plausible. An alternative explanation, which is entirely without experimental support, is that there may be in serum a copper

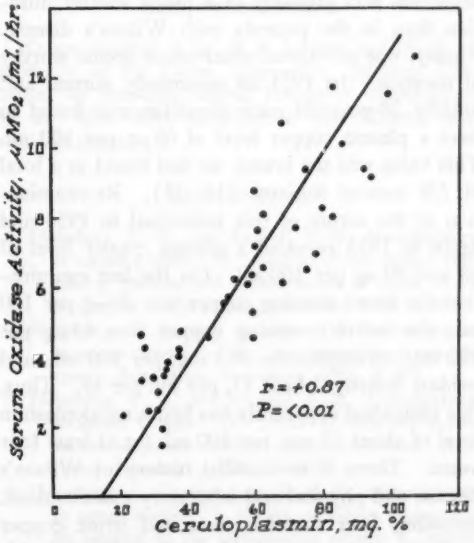

FIG. 3. THE CORRELATION BETWEEN THE SERUM OXIDASE ACTIVITY AND CERULOPLASMIN

For explanation see legend for Figure 1.

protein or proteins with copper concentrations of less than 0.32 per cent which are closely related to ceruloplasmin and which react with this substance. If such were the case, it might explain why there is a poor correlation between the immunologically determined ceruloplasmin concentration and the clinical manifestations of Wilson's disease.

The significance and importance of low levels of ceruloplasmin need to be elucidated. Scheinberg and Gitlin (15) have advanced the hypothesis that Wilson's disease is due to a congenital deficiency of ceruloplasmin. This hypothesis is indeed attractive and has been favored by Bearn (24) as well as by ourselves (13). If hypoceruloplasminemia is in itself of pathogenetic significance, it might be expected that there would be a correlation between the degree of deficiency of ceruloplasmin in the serum and the duration or severity of the clinical manifestations of Wilson's disease. In our small group of patients the ceruloplasmin levels ranged from 2 to 19 mg. per 100 ml. and no such correlation was observed. Furthermore, ceruloplasmin levels as low as those found in Wilson's disease were observed in three patients with the nephrotic syndrome. Admittedly, the hypoceruloplasminemia in the cases of nephrosis was probably of a much shorter duration than in the patients with Wilson's disease. Finally, one additional observation seems worthy of mention. In 1951 an apparently normal and healthy 28-year-old male physician was found to have a plasma copper level of 68 μg per 100 ml. This value was the lowest we had found in a total of 228 normal subjects (14, 23). Re-examination of the serum of this individual in 1954 and again in 1955 revealed a plasma copper level of 68 and 69 μg per 100 ml. On the last examination the direct-reacting copper was 26 μg per 100 ml., the indirect-reacting copper was 43 μg per 100 ml., ceruloplasmin 20.3 mg. per 100 ml., and oxidase activity 2.4 μM O_2 per ml. per hr. Thus, this individual apparently has had a ceruloplasmin level of about 20 mg. per 100 ml. for at least four years. There is no familial history of Wilson's disease and physical and laboratory examinations, including liver function tests and urine copper analysis, are unremarkable except for palpable liver and a persistent unexplained neutrophilic leukocytosis. Although this individual is not entirely normal, it seems most unlikely that he has hepatolenticular degeneration. Yet, for a period of at least four years, he has had a ceruloplasmin concentration only about 1 mg. per 100 ml. higher than patient D. C. (Table VI) with severe disease.

The low levels of ceruloplasmin found in Wilson's disease might be explained by either failure to form or increased destruction of this enzyme. In the nephrotic syndrome loss of this protein in the urine seems to be the most important factor. In a previous publication from this laboratory (14) it was pointed out that patients with the nephrotic syndrome excrete between 100 and 600 μg of copper per 24 hours in the urine. The degree of cupriuria was found to be correlated with the degree of proteinuria and it was postulated that ceruloplasmin was being excreted by the kidney under these circumstances. Our present observation that 46 to 75 mg. of ceruloplasmin were excreted daily in the urine of five patients with the nephrotic syndrome gives support to this suggestion. In these cases the ceruloplasmin accounted for 60 to 80 per cent of the copper excreted in the urine per day. Since the normal serum pool of ceruloplasmin is approximately one gram, these patients were excreting about one-twentieth of the normal serum pool of this protein in the urine per day. This represents an appreciable loss of ceruloplasmin from the body by an abnormal route, since ceruloplasmin is not found in the urine of normal subjects in detectable quantities. Apparently, in those cases of the nephrotic syndrome in which hypocupremia and hypoceruloplasminemia were found, the rate of synthesis of ceruloplasmin was not great enough to maintain normal levels of ceruloplasmin.

The results of our tissue analyses for ceruloplasmin are of interest but difficult to interpret. Both in the control subjects and in the case of Wilson's disease ceruloplasmin could be demonstrated only in liver and in kidney. The finding of ceruloplasmin in the kidney in concentrations equal to or higher than in the liver was unexpected. Its absence in tissues, such as basal ganglion, cerebellum and erythrocytes, which normally contain greater quantities of copper than the kidney, is noteworthy. It is also interesting that the proportion of the copper in the liver and kidney of the case of Wilson's disease which could

be accounted for as ceruloplasmin was extremely small.

SUMMARY

1. Total serum copper, direct-reacting copper, indirect-reacting copper, ceruloplasmin, and oxidase activity were measured in the sera of 10 *normal* subjects. The mean values (± one standard deviation) observed were as follows: total serum copper, 108 ± 9 μg per 100 ml.; direct-reacting copper, 5 ± 6 μg per 100 ml.; indirect-reacting copper, 103 ± 11 μg per 100 ml.; ceruloplasmin, 34 ± 4 mg. per 100 ml.; oxidase activity, 3.9 ± 0.7 μM O_2 per ml. per hr.

2. Corresponding values in 10 pregnant women in the last trimester of *pregnancy* were as follows: total serum copper, 257 ± 38 μg per 100 ml.; direct-reacting copper, 29 ± 13 μg per 100 ml.; indirect-reacting copper 228 ± 35 μg per 100 ml.; ceruloplasmin, 84 ± 15 mg. per 100 ml.; oxidase activity, 9.6 ± 2.2 μM O_2 per ml. per hr. A high degree of correlation was observed between the total serum copper, indirect-reacting copper, ceruloplasmin, and oxidase activity.

3. Mean values in seven patients with *chronic infections* were as follows: total serum copper, 192 μg per 100 ml.; direct-reacting copper, 17 μg per 100 ml.; indirect-reacting copper, 175 μg per 100 ml.; ceruloplasmin, 68 mg. per 100 ml.; oxidase activity, 8.1 μM O_2 per ml. per hr. As in the pregnant women, a high degree of correlation was observed between the total serum copper, indirect-reacting copper, ceruloplasmin, and oxidase activity.

4. In nine patients with the *nephrotic syndrome*, the total serum copper values ranged from 129 μg per 100 ml. to as low as 36 μg per 100 ml. In those in whom there was hypocupremia, a corresponding reduction in the ceruloplasmin concentration was present. Four of these patients were found to excrete between 46 and 75 mg. of ceruloplasmin per 24 hours in the urine.

5. In 14 patients with *hepatolenticular degeneration*, the total serum copper values ranged between 35 and 100 μg per 100 ml. Ceruloplasmin values were below normal, ranging between 2 and 19 mg. per 100 ml. In general, there was an increase in the direct-reacting fraction of copper and a decrease in the oxidase activity. There was little correlation between the total serum copper values and the ceruloplasmin values. In five patients there was good correlation between the indirect-reacting copper and ceruloplasmin. No correlation was found between the ceruloplasmin level and the duration or severity of the clinical manifestations of the disease. Significant quantities of ceruloplasmin were not found in the urine.

6. In neurologic diseases other than hepatolenticular degeneration the total serum copper level was either normal or increased and there was no decrease in the quantity of ceruloplasmin in the serum.

7. Ceruloplasmin was found in small quantities in the liver of one "control" subject and in the kidney of two "control" subjects. Ceruloplasmin was not found in brain, spleen, bile, or erythrocytes of the "control" subjects or of the patient with Wilson's disease. Ceruloplasmin was found in the liver and kidney of one patient with Wilson's disease but this protein represented a much smaller proportion of the total copper demonstrable in these tissues than was the case in the control subjects.

8. The copper protein in normal erythrocytes was found to be immunologically distinct from ceruloplasmin.

ACKNOWLEDGMENTS

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ERYTHROCYTE PRESERVATION. V. RELATIONSHIP BETWEEN CHEMICAL CHANGES AND VIABILITY OF STORED BLOOD TREATED WITH ADENOSINE[1]

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Blood refrigerated in acid-citrate-dextrose is known to undergo marked derangement in carbohydrate metabolism and loss of viability during storage. Previous studies of the "storage lesion" have indicated that this metabolic deterioration is arrested once the red cell is introduced into the active circulation of the recipient, and that the ability of the red cells to withstand further *in vitro* storage is regained (1).

The addition of adenosine to stored blood has been shown to produce a repletion of cellular organic phosphates and an increased ability of the cells to oxidize glucose aerobically (2). Further data will be presented in this paper on the reversal of other storage changes and in particular the improved maintenance of the post-transfusion viability of the erythrocyte.

MATERIALS AND METHODS

Blood was stored routinely at 4° in acid-citrate-dextrose (National Institutes of Health, Formula B). Measurements of the phosphate partition of red cells, concentration of adenosine, and adenosine uptake by red cells were performed as reported previously (2). Osmotic fragility of erythrocytes was measured by the technique of Shen, Ham, and Fleming (3). Potassium was determined on whole blood and plasma with the use of the Baird flame photometer.[2]

Post-transfusion survival of erythrocytes was estimated with the use of a double isotope technique (4). Stored cells were labelled with Cr^{51}, and a measured amount of those cells of known radioactivity was injected. Simultaneously, the blood volume of the recipient was determined with fresh P^{32}-tagged cells. The total counts of Cr^{51} injected divided by the blood volume gave the theoretical activity in counts per minute per milliliter in the recipient's blood. Post-transfusion samples at 1, 2, 3, 5, and 7 days were obtained for the determination of Cr^{51} activity. Since excessive destruction is limited to the first 48 hours and the slope thereafter approximates 1 per cent per day destruction, further samples were felt to be unnecessary. The ratio of radioactivity in the samples to the theoretical level of activity was plotted graphically and extrapolated to zero time. This value was designated per cent survival. With the use of this procedure, fresh rabbit and human blood have a survival in compatible recipients of 90 per cent. The initial loss is ascribed in part to washing of the cells, since unwashed fresh erythrocytes have 96 per cent survival.

P^{32} activity was determined with the use of a helium-filled Geiger tube. The counting efficiency of P^{32} was 20 per cent, and the background was 23 cpm. Cr^{51} was measured in a well of a scintillation counter separated from the crystal by a 3/128 inch brass shield. Counting efficiency of Cr^{51} was 3 per cent with a background of 80 cpm. Cross counting of Cr^{51} with the Geiger tube was less than 1 per cent, and P^{32} counts on the scintillation counter were less than 3 per cent of those obtained with the Geiger tube.

RESULTS

Studies to define more completely the effect of adenosine on the storage lesion have been conducted on both rabbit and human erythrocytes. The following experiments have been carried out repeatedly to assure the reproducibility of the phenomenon and its implications in terms of cell viability.

I. *Rabbit blood*

Rabbit blood, which had been collected in ACD and stored at 4° for three weeks, was divided into two aliquots. Adenosine (2,000 μM per 100 ml. RBC) was added to the first aliquot and saline to the other which served as a control. Both samples were then incubated for one hour at 37° and stored thereafter at 4° for three weeks additional,

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[2] We are indebted to the laboratory of Dr. Belding H. Scribner for these analyses.

TABLE I

Chemistry and survival of stored rabbit blood after adenosine treatment

Rabbit blood was stored at 4° in ACD for 20 days at which time it was divided into two aliquots: 1) Control with saline addition; 2) Adenosine (2,000 μM per 100 ml. RBC) added. Both samples were incubated at 37° for one hour and returned to 4° refrigeration for subsequent storage.

Days' storage	μM P/100 ml. RBC			Osmotic fragility peak (per cent NaCl)*	Post-transfusion survival (*per cent*)	
	Inorganic	Organic	Increase in organic			Av.
0 day	173	2,408		0.48		
20 day	1,443	1,018		0.54	77, 77	77
20 day; 1 hr. rejuvenated:						
Control	1,007	911		0.60		
Adenosine	698	1,665	754	0.52		
27 day; 7 day rejuvenated:						
Control	1,625	601		0.60	65, 66	66
Adenosine	279	2,131	1,530	0.44	79, 80	80
34 day; 14 day rejuvenated:						
Control	1,688	542		0.60	53, 53	53
Adenosine	338	1,951	1,409	0.48	72, 77	75
41 day; 21 day rejuvenated:						
Control	1,741	443		0.62	32, 36	34
Adenosine	694	1,657	1,214	0.48	60, 70	65

* Each value represents the concentration of NaCl at which there is the maximum tendency for the greatest number of cells to hemolyze. The fragility peak is obtained from a curve plotted with $\frac{\Delta\% \text{ hemolysis}}{\Delta\% \text{ NaCl}}$ as abscissa and % NaCl as ordinate (1).

thus representing a total storage time of 6 weeks. In Table I, are recorded the data from one such experiment. During the first three weeks of storage, the organic phosphates of the red cell had decreased about 1400 μM per 100 ml. RBC, the red cell fragility to hypotonic saline had increased, and the post-transfusion survival had declined to 77 per cent. After one hour of incubation, the cells treated with adenosine exhibited considerable resynthesis of organic phosphates and improvement in osmotic fragility. Although the initial uptake of 65 per cent of adenosine by the cells was essentially unchanged during the following week of storage at 4°, there was further synthesis of organic phosphates. Subsequent storage showed a gradual decline of organic phosphates in the adenosine-treated cells, but the content of phosphorylated compounds exceeded greatly that found in the untreated cells. The erythrocytes were less fragile in hypotonic saline after adenosine treatment.

Post-transfusion survival measurements were carried out in two recipient animals for each sample tested. As anticipated, the control blood at the 4th, 5th, and 6th week of storage decreased in viability from 66 to 53 to 34 per cent. During this same time the adenosine-treated blood showed a total decrease of only 12 per cent, that is, a fall from 77 to 65 per cent.

II. *Human blood*

A similar study was carried out on human blood, collected in ACD and stored at 4°. On the

TABLE II

Chemistry of stored human blood after adenosine treatment

Human blood was stored at 4° in ACD for 21 days and then divided into two aliquots: 1) Control with saline addition; 2) Adenosine (2,500 μM per 100 ml. RBC) added. Both samples were incubated in a water bath at 37° for one hour and returned to 4° refrigeration for subsequent storage.

Days' storage	μM P/100 ml. RBC		Osmotic fragility peak* (per cent NaCl)	K *mEq./L. RBC*
	Inorganic	Organic		
0	125	1,430	.42	
21	892	435	.50	
21, 1 hr. rejuvenated:				
Control	556	480	.50	56.9
Adenosine	71	1,373	.44	68.1
28, 7 days rejuvenated:				
Control	693	366	.52	49.4
Adenosine	64	1,031	.44	55.2
42, 21 days rejuvenated:				
Control	817	273	.54	38.3
Adenosine	326	1,213	.48	50.4

* See legend, Table I.

21st day of storage, the blood was divided into two aliquots and after saline solution was added to the control and adenosine (2,500 μM per 100 ml. RBC) to the second, the aliquots were incubated for one hour and refrigerated. In general, the results of the chemical analyses (Table II) were similar to those observed with rabbit erythrocytes. The uptake of adenosine was 63 per cent after the one-hour incubation period and 68 per cent the following week. The cellular organic phosphates, particularly the easily hydrolyzable and non-hydrolyzable fractions, were increased after incubation with adenosine and were maintained well throughout the additional storage period. Osmotic fragility and intracellular potassium of the adenosine-treated erythrocytes reflected a similar improvement as compared with the control.

The viability of erythrocytes treated with adenosine showed essentially no change over the three weeks following incubation, while the control blood in acid-citrate-dextrose lost viability rapidly (Table III). Thus, at the total storage time of six weeks, the post-transfusion survival of ACD blood was 15 per cent, while the blood treated with adenosine was 87 per cent viable.

DISCUSSION

Stored in ACD at 4° the red cell gradually undergoes a multiplicity of changes. Some or all of these changes may be presumed to have a direct bearing on the loss of viability of the cell in the sense of the cell's capacity to maintain itself in active circulation after transfusion and perform its oxygen-carrying functions. It has been established by previous workers in this field that while several of these *in vitro* tests may show a high degree of correlation with the viability of cells after transfusion, this is not invariably the case and that no *in vitro* test can be employed as a dependable reflection of the exact viability of the erythrocyte.

Cells damaged during storage in ACD are removed from circulation within the first 24 to 48 hours after transfusion. The slope of the survival curve after 24 hours of the treated transfused cells in these studies was similar to that observed with ACD blood followed four weeks. It is believed, therefore, that any damage incurred by storage to the cells with added adenosine was completely reflected by the measurements made during the first seven days after transfusion.

It is well known that the erythrocyte is dependent upon carbohydrate metabolism to meet certain energy requirements if viability is to be maintained. At 4°, viability is lost rapidly if glucose is removed, or if inhibitors such as fluoride or oxalate are added. Thus in these studies in which attention has been focused on the red cell phosphate partition as a metabolic indicator, the marked improvement in this system observed after the incubation of stored blood with adenosine was considered indicative of improvement in the preservation of the erythrocyte.

The data presented in this report emphasize the inter-relationship between the energy potential of the red cell and viability. Adenosine not only restores organic phosphorylated compounds, but the osmotic resistance and the potassium content as well. Post-transfusion survival studies clearly indicate that these alterations have been reflected in improved preservation of blood.

TABLE III

Survival of stored human blood after adenosine treatment **

Days' storage	Post-transfusion survival (*per cent*)	
		Av.
28, 7 days rejuvenated:		
Control	47, 53, 68	56
Adenosine	67, 69, 82	73
42, 21 days rejuvenated:		
Control	14, 15, 17	15
Adenosine	83, 88, 90	87

* Experimental details as given in Table II.

SUMMARY

The reversal of the storage lesion with adenosine has been demonstrated in rabbit and human blood stored at 4° C. in ACD and has been characterized by *in vitro* changes and by the effect on post-transfusion survival.

The incubation of three-week stored blood with adenosine for one hour at 37° C. resulted in the regeneration of organic phosphates, improvement in osmotic fragility, and increase in intracellular potassium. These changes were sustained for a subsequent three-week interval at refrigeration temperature.

There was an associated improvement in post-transfusion viability of adenosine-treated cells over the second three-week period as compared with control ACD blood.

The evidence would seem to indicate that a diminished energy potential of the cell is a primary factor in the deterioration of the erythrocyte under these conditions of storage.

ACKNOWLEDGMENT

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DETAILED ELECTROPHORETIC ANALYSES OF SERA OF HEALTHY YOUNG MEN[1]

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The electrophoretic patterns of plasma and serum obtained from healthy individuals have been described by a number of investigators and the results of such analyses are summarized in a recent paper (1). Appreciable fluctuations in the concentrations and percentage distributions of the major protein components are noted. A critical examination of these data shows that workers in this field use a variety of buffer solutions with different ionic strength and usually average the results obtained for males and females in different age groups. Furthermore, Bernfeld, Donahue, and Homburger (2) have observed large variations in the general appearance of electrophoretic patterns and have emphasized the necessity of considering the finer structures of the contour in the analytical procedure.

The data obtained from electrophoretic analyses depend on the method by which the area of the electrophoretic pattern is subdivided. The analysis of electrophoretic patterns of serum proteins is generally done by the procedure of Tiselius and Kabat (3) which involves drawing a perpendicular from the base line to the minimum between two peaks, or the components may be resolved by drawing a series of symmetrical curves as described by Svedberg and Pedersen (4). The method of Tiselius and Kabat has the advantage of simplicity and the results approach those given by the Pedersen procedure (5, 6). Wiedemann (7) subdivided the electrophoretic areas of sera with the aid of Gaussian curves and stated that nine components could be distinguished.

Because of the wide discrepancies in values for normal plasma and serum, it was of interest to analyze in detail electrophoretic patterns which were obtained under carefully controlled conditions. This paper presents the results of electrophoretic analyses of sera from healthy young men.

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METHODS

First year, male, medical students, ranging in age from 20 to 25 years, served as subjects during the period from February 1 to May 1, 1952. These students had finished their final examinations in Anatomy on January 30. The subjects were selected at random and no attempt was made to pick any individual at any given time. In several cases, brothers who were in the College were used as subjects. Care was taken to avoid obtaining blood from individuals with respiratory, alimentary or other disorders. Blood was drawn before breakfast and the serum was separated within two hours. Serum (0.85 ml.) was diluted with 2 ml. barbiturate buffer (pH $8.6 \pm .02$, ionic strength 0.1) and dialyzed in the cold against three changes of the buffer solution during a period varying from 18 to 30 hours. Electrophoresis was done in a Klett Model of the Tiselius apparatus using a microcell of 2 ml. capacity. The temperature of the bath was maintained at $2.0 \pm 0.01°$. Electrophoresis was allowed to proceed at 7 ma. (10 v per cm.) for about 100 minutes until the distance between albumin and the δ-boundary approximated fixed positions marked on a celluloid sheet at the viewing screen. The picture was taken by Longsworth's scanning technique (5) immediately after the current was shut off. The conditions of illumination, dilution of the serum, dialysis, buffer solution and exposure of the plate were carefully controlled. The ascending patterns were analyzed. Tracings of the pattern, magnified four times, were made on coordinate paper and included the base and the edges of the picture. The position of the pattern in the enlarger was always the same and precautions were taken to avoid parallax. When necessary, corrections for the defects of the optical systems were made from the respective base lines.

Ordinarily, the absolute mobilities of protein components in an electrophoretic pattern are determined by measuring the displacements from the starting boundary. The accuracy of such determinations depend on protein concentration (8), the time of electrophoresis, absence of leaks in the system, electro-osmosis, density inversion, and the influence of conductivity and pH gradients in the solution during electrophoresis. The relative influence of some of these factors on the migration and concentration gradient of a component cannot be properly evaluated; however, variations are minimized by carefully controlled experimental procedures. Values for absolute mobilities of individual components, obtained under routine conditions, show considerable variations. On

TABLE I

Relative rates of migration of mean boundary positions and their respective leading limits at base line

	Alb.	α_1^0	α_1^1	α_1^2	α_1^3	α_2^1	α_2^3	β^1	β^2	β^3	β^4	τ	γ_1	γ_2	γ_3	γ_4	γ_5	
Boundary	6.00	5.56	5.36	5.12	4.93	4.36	3.95	3.63	3.34	3.05	2.75	2.45	2.13	1.79	1.48	1.22	1.02	
Limit		5.68	5.68	5.36	5.43	4.88	4.43	3.82	3.67	3.42	3.05	2.75	2.45	2.13	1.79	1.50	1.39	.646*

* Trailing limit of γ_5.

the other hand, the values for relative mobilities of these components are reproducible within narrow limits.

Careful analyses of the electrophoretic patterns of a large number of sera and plasmas from healthy and diseased individuals indicated that at least 17 components are present. The relative mobilities of the mean positions and the leading and trailing limits of each component were determined. The value of 6.00 was arbitrarily assigned to albumin, since it approximates the order of the absolute mobility. The remaining values were proportioned to their relative migration rates (Table I).

The following basic assumptions were made for the analysis: (a) all boundaries were considered to be symmetrical; (b) areas responsible for dissymmetry of a boundary were attributable to the presence of another protein; (c) the relative positions of the mean boundaries and their contour "limits" approaching the base line remained constant; and (d) the relative migration rates were independent of protein concentration.

The following procedures are described for delineating the areas of these components. The position where the trailing edge of the γ_5-globulin boundary apparently ceases its contribution to the δ-area was designated as A (Figure 1) and represents a point where the contour visibly deviates from the tangent drawn through the point of inflection. The distance between point A and the albumin boundary axis was accurately measured with a metal ruler. From this measurement and from relative mobility values (Table I) for the albumin axis (6.00) and point A (.646), the zero time position and the positions of the remaining components were readily calculated. The position of the axes of the boundaries and their limits on the base line were accurately marked with a sharp needle on the pattern and all axes and boundaries drawn as thin sharp lines. Needle dividers were used for transposing and for checking the ordinates.

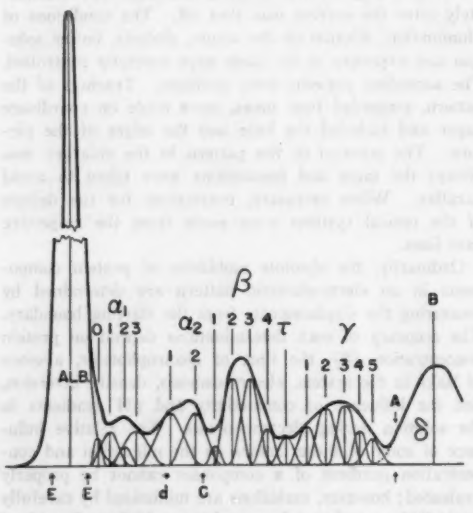

FIG. 1. THE ELECTROPHORETIC PATTERN AND 17 COMPONENTS CONSTRUCTED FROM MEAN VALUES OF 46 SERA

The significance of symbols are described in text.

It was found by experience that the analysis of the envelope was most easily accomplished by using the contour ordinates of the limits of α_2^3- and α_2^2-. The details of the analysis and the construction of curves for these two components are shown in Figure 2. The boundaries of these two components were drawn so that the sum of the ordinates of the contributing boundaries equaled the height of the envelope at every point. It is essential that a number of points be used in transposing a segment of a curve.

As a rule, the boundaries for β^1-, β^2-, β^3- and the leading half of β^4-globulins were next resolved. After these analyses, the γ-globulins were resolved by using the trailing limit of γ_5-globulin as a starting point. If the α_2- and β-globulins and the γ-globulins are properly analyzed, the trailing edge of the β^4-globulin should be symmetrical to its leading edge, indicating that the positions and differentiation of the respective components are correct. The albumin boundary was obtained by reflecting points on the leading edge about its axis. The residual area between the reflected line of albumin and the envelope contains the α_1^0-component and some of the α_1^1-globulin. The resolution of the α_1-globulins completed the analysis of the pattern. The fixed positions of the components determine the solution of the envelope and prevent cumulative errors. The areas of the components were measured at least twice by planimetry. The difference of the sum of the component areas and the total area of the pattern (albumin through γ_5-globulin) should not differ by more than 2 per cent.

Two points (E in Figure 1) obtained by extending the tangent of the slope of the upper third of the albumin envelope to the base line were found to be useful in evaluating the electrophoresis technique. The distance of these points from the albumin axis is uniform with good technique. An increase in the spread of these points, due to a broadening of the albumin boundary,

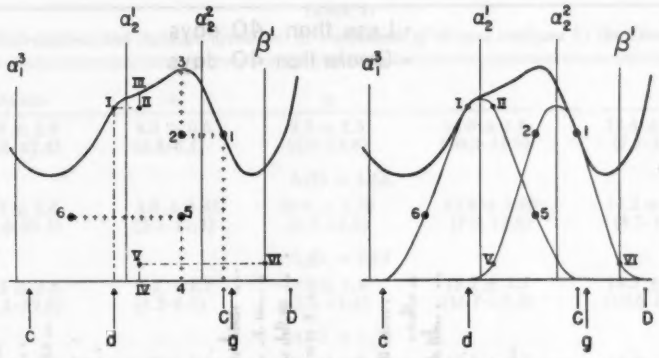

FIG. 2. DETAILS OF ANALYSIS AND CONSTRUCTION OF CURVES FOR α_2^1- AND α_2^2-GLOBULINS

The following symbols are used: *c* and *C* and *d* and *D* represent the leading and trailing limits of the α_2^1- and α_2^2-globulins, respectively. *g* is the leading limit for β^1-globulin. Point *I* on the envelope is a projection of *d* and point *II* is obtained by reflection of point *I* about the α_2^1 axis. The distance *II–III* is transposed to the base line and is shown as *IV–V*. Point *VI* represents a reflection of point *V* about the α_2^2 axis. In a similar manner, point *C* is projected to the envelope (point *1*) and the transposition of points is done as described above.

The curve for α_2^1-globulin is constructed from points *6*, *I*, *II* and *5* with *c* and *C* as limits; α_2^2-globulin is drawn through points *V*, *2*, *1* and *VI* with *d* and *D* as limits. The line between *6* and *I*, for example, is slightly curved and the curvatures near the limits and the peaks change gradually.

is usually caused by a poor starting boundary or by a delay in taking the picture after the current is stopped.

RESULTS

The individual planimeter values, which were found to give the best indices for relative concentrations, were plotted against days following the examination period. It was observed that the α_1^3, γ^1- and γ^2-globulin values were higher during the first 25 days than during the subsequent period. The α_2^1-globulin concentrations were low during the first 40 days and subsequently increased; in contrast, the α_2^2-values were high during this same preliminary period and decreased after this time. Since the most pronounced changes were noted for the α_2^1- and α_2^2-globulins, the data were separated to cover the first 40 days (group 1) and the 41 to 92-day period (group 2) and presented in Figure 3. The mean and the variance for each component were not presented because of the skewed distribution of the data. The relationship between α_2^1- and α_2^2-globulins could be best demonstrated by plotting the ratios of these two components in individual sera against days after examination (Figure 4).

The envelope and the 17 components of an electrophoretic pattern (Figure 1) were constructed from the mean values of the respective areas of 46 sera in group 2.

The electrophoretic patterns of the sera in group 2 were also analyzed by the Tiselius-Kabat method (3). The mean values for the percentage distribution, the standard deviations and the range of values are shown in Table II; additional data for sera, analyzed in barbiturate buffer at pH 8.6, are included for comparison. The values published by Sterling (11) for 10 young men agree closely with the data obtained in the present experiments. The differences in the mean values and the variations about the mean, observed by other investigators, are probably attributable to differences in technique, and to the influence of sex and age on the plasma protein composition.

Influence of family relationship

Electrophoretic patterns of sera from identical twins, seven pairs of brothers differing from 1 to

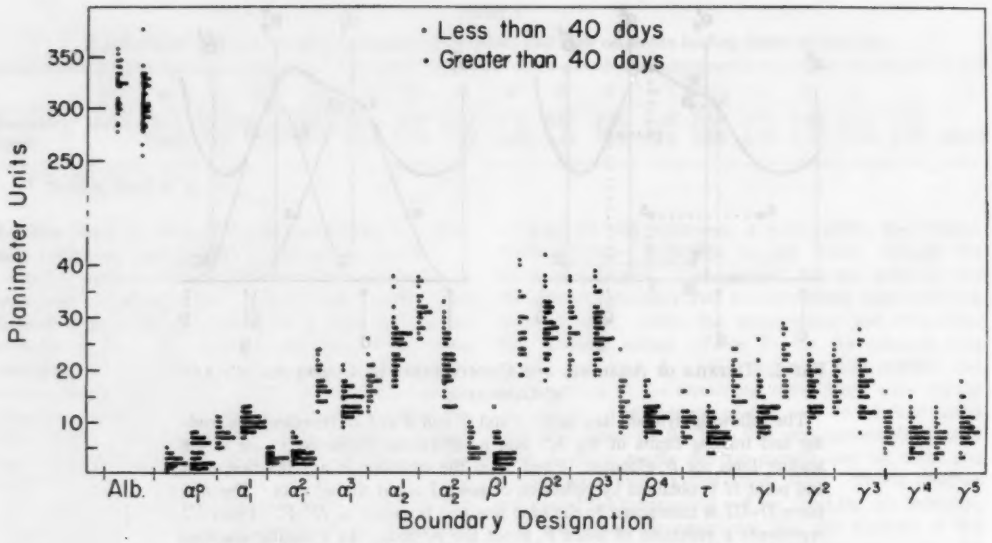

FIG. 3. INDIVIDUAL CONCENTRATIONS OF 17 COMPONENTS OF SERUM

3 years in age and a father and son were analyzed. In all cases, samples of blood were drawn from each pair within a few minutes of each other.

Tracings of the electrophoretic patterns for six pairs of brothers are shown in Figure 5. The patterns of the sera from respective brothers show a striking similarity in size and shape of the major components; slight differences in the shape of boundaries may be partly due to the extent of migration of the pattern. A comparison of these patterns and the analytical data between unrelated individuals shows considerable differences.

Two sets of analytical data for identical twins, representing sera obtained two years apart, show that most of the values are in close agreement (Figure 6). Data for five of the seven brothers and a father and his son are presented in Figure 7. The results for the remaining two brothers, which were omitted due to lack of space, were similar to the data presented. The values for R. W. and W. W. are almost identical except for one component. In the remaining cases, there is no consistent pattern in the type of component or the differences in concentrations between components

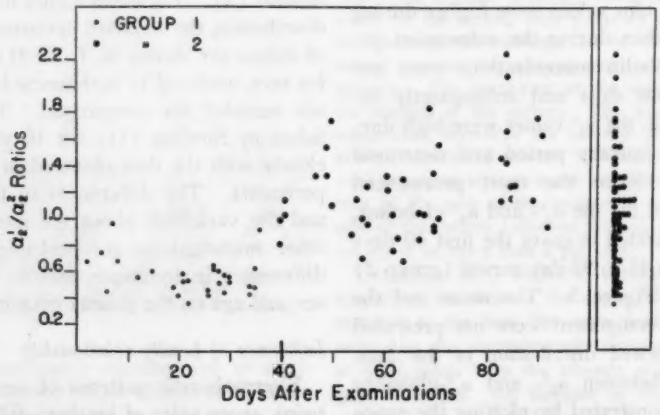

FIG. 4. THE α_2^1/α_2^2 RATIOS AT VARYING PERIODS AFTER EXAMINATIONS

TABLE II

Mean percentage distributions and standard deviations of components of 46 sera analyzed by the Tiselius-Kabat method *

No. sera	Albumin	α_1	α_2	β	γ	Reference
46	60.6 ± 3.5 (53.4–67.6)	4.0 ± 0.6 (2.8–6.1)	9.2 ± 1.5 (6.0–12.8)	14.0 ± 1.6 (10.2–17.9)	12.4 ± 2.7 (7.5–21.2)	Authors
			A/G = 1.54			
43	53.3 ± 2.6 (47.6–59.2)	8.0 ± 1.35 (5.3–11.5)	10.4 ± 1.38 (7.7–12.9)	13.8 ± 1.89 (7.9–17.6)	14.2 ± 2.68 (8.7–19.7)	(9)
			A/G = 1.15			
10	56.5 ± 2.8 (53.1–59.8)	5.2 ± 1.7 (4.2–6.0)	9.0 ± 1.4 (7.8–11.4)	15.2 ± 1.5 (13.7–17.2)	14.2 ± 1.8 (10.6–16.2)	(10)
			A/G = 1.30			
10	60.3 ± 2.8 (55.0–64.8)	4.0 ± 0.8 (2.5–5.3)	9.7 ± 1.5 (7.3–12.9)	12.8 ± 1.2 (9.9–14.3)	13.2 ± 1.7 (10.4–16.1)	(11)
			A/G = 1.52			
80	56.2 ± 2.8	7.2 ± 1.3	8.8 ± 1.9	13.1 ± 2.5	14.7 ± 2.6	(12)

* Figures in parentheses represent range in values.

for the respective brothers. It will be noted that the shapes and concentrations of the γ-globulins are quite similar for each pair of brothers but may differ considerably for unrelated individuals. The phenomenon is particularly striking for the father and son.

DISCUSSION

The Tiselius-Kabat procedure for analyzing electrophoretic patterns does not consider the contour or the effect of the distance of migration (5). Results of such analyses may be misleading, since areas (concentrations) of major components of sera may be similar despite differences in the shapes of the envelopes of the patterns (2). The analytical procedure presented in this paper makes it possible to determine the material responsible for changes in the contour of the pattern. The analysis is time consuming but the results are precise. Duplicate analyses of a serum show that the

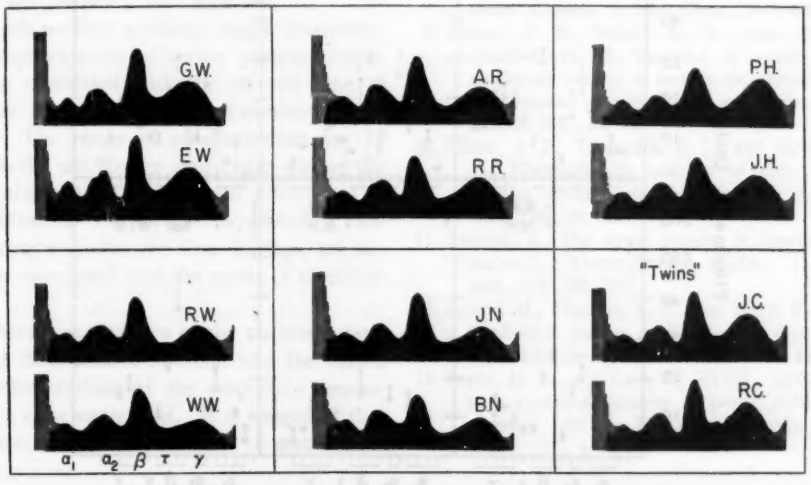

FIG. 5. ELECTROPHORETIC PATTERNS OF SIX PAIRS OF BROTHERS

The solid area represents the globulins and the shaded areas represent the trailing portion of albumin and the leading portion of the δ-boundary.

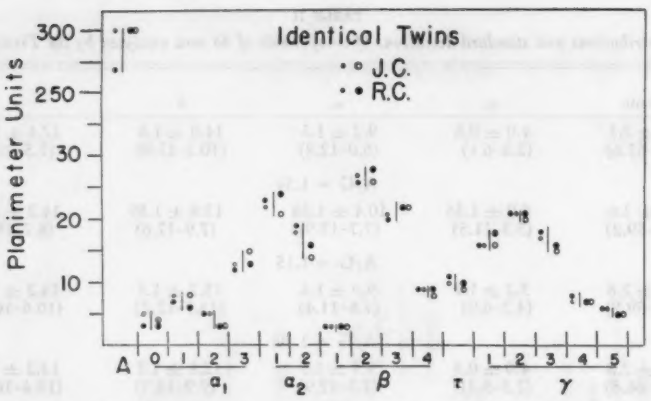

FIG. 6. THE CONCENTRATIONS OF PROTEIN COMPONENTS OF SERA FROM IDENTICAL TWINS

The samples were obtained in February, 1952 (small circles) and February, 1954 (large circles).

concentration for each component is reproducible. This procedure could be particularly useful for determining the finer details in patterns of sera which are being studied during the course of observations in healthy or diseased individuals.

Examination of the contours in Figure 1 will indicate that the variations in values for absolute mobilities obtained by using the peak or the centroidal ordinate of a boundary may be attributed to a shift of the maxima of the peaks due to changes in the concentrations of the components within given areas.

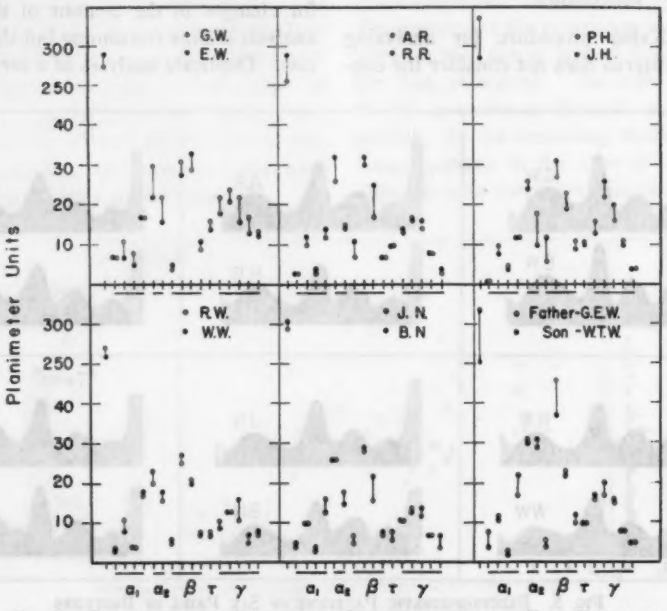

FIG. 7. COMPARISON OF COMPONENT CONCENTRATIONS IN FIVE PAIRS OF BROTHERS AND A FATHER AND SON

Since the factors which might affect the ratio of the α_2^1- to α_2^2-globulins in sera during and after the first 40 days following examinations (Figure 4) are unknown, conclusions concerning the significance of these changes would not be justified. This shift in the concentration of these 2 components occurs early in March and may be associated with climatological conditions. It is suggested, however, that the stress of the examination is responsible for the changes observed.

The consistent finding in this study that the contour of the electrophoretic patterns and the concentrations of the protein components of sera in brothers are similar would indicate that there is a hereditary basis for these observations. It is noteworthy that abnormal globin of sickle cell hemoglobin is transmitted by genes. Although, it may not be directly pertinent to this problem, it is interesting that a strong similarity in the excretion of nitrogenous metabolites was observed between certain siblings (13).

SUMMARY

A method for dividing the electrophoretic pattern of serum into 17 components is described. The procedure is based on the assumption that each component is symmetrical about its axis and has a fixed relative mobility value. Precautions for accurate electrophoretic analysis and for delineating each boundary are discussed.

In 73 male medical students, single determinations of concentrations of serum protein components were correlated with the elapsed time of about three months after final examinations in Anatomy. The range of concentrations for 15 components did not change appreciably during the period of observation. Significant differences in the concentrations of the two α_2-globulins, observed during and after the first 40 days, are believed to be associated with the stress of examination.

In brothers, the contours of the electrophoretic patterns of their sera are similar and the values for the concentrations of the respective components are in close agreement. It is suggested that the concentration and distribution of serum proteins of young healthy individuals are under genetic control.

The significance of the analytical procedures and the results are discussed.

ACKNOWLEDGMENT

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THE RENAL CLEARANCE OF ALKALI-STABLE INULIN

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Commercial inulin available at present is not a homogeneous product (1). It consists of two major fractions the proportions of which vary: (a) a portion of relatively large molecular weight resistant to heating in alkali; and (b) a portion of smaller molecular weight susceptible to destruction with alkali. In addition, most samples contain a small percentage of fructose, which also yields color in the analytical methods for inulin unless removed by treatment with yeast or hot alkali.

Two recent reports (2, 3) indicate that the clearance of the alkali-stable and alkali-labile fractions of inulin may not be equal. Since some commercial preparations of inulin (such as the one used in this study) may contain as much as 50 per cent of the alkali-labile material, considerable errors in estimation of glomerular filtration rate would occur if a difference in clearance of the two fractions exists.

The present report indicates that the clearance of the two fractions is identical, within the limits of error of analysis, in confirmation of the preliminary report by Cotlove (1).

METHODS

Subjects. The subjects were patients of the National Heart Institute at the Clinical Center. One was a normal volunteer. The others had renal or endocrine diseases. In most cases studies were performed in the fasting state. The comparisons were made during clearance determinations performed for other purposes.

Procedure. Before each clearance determination, the ampoules of inulin to be used were mixed together and a portion withdrawn for analysis.[1] A priming injection and a sustaining infusion of inulin were administered intravenously. The plasma level of the alkali-stable fraction was approximately 13 mg. per cent. After a 30 to 40-minute equilibration period, 3 to 8 urine collection periods were obtained, each of 20 to 40 minutes' duration. Blood for analysis was obtained in a syringe containing heparin at the midpoint of each period.

Analytical methods. Alkali-stable inulin in plasma and urine was determined by a modification of published methods (4–7) as follows: protein-free plasma filtrates were prepared according to Somogyi (8) by adding 7 ml. of water, 1 ml. of $ZnSO_4$ reagent,[2] and 1 ml. 0.75 N NaOH (dropwise and with constant mixing) to 1 ml. of plasma. After 20 minutes, during which the flasks were shaken occasionally, the solutions were centrifuged and the supernatant decanted.[3] Duplicate 2 ml. aliquots of supernatant, appropriately diluted urine and standards were pipetted into 20 × 150 mm. tubes. Five-tenths ml. of 4N NaOH was added from a 1 ml. Krogh-Keys syringe-pipette. The tubes were covered with marbles and placed in a 100° C. bath for 20 minutes.[4] After cooling, 6.25 ml. of diphenylamine reagent[5] were added from a 10 ml. Krogh-Keys syringe-pipette. Mixing was unnecessary if the reagent was injected vigorously. The marbles were replaced and the tubes were reheated in the 100° bath for 30 minutes,[6] cooled, and read at 620 mμ in a Coleman Jr. spectrophotometer.

"Total" inulin was determined according to the method of Harrison (6) as modified by Kennedy and Kleh (7). One ml. samples of plasma or diluted urine were treated with yeast; plasma protein was precipitated with $ZnSO_4$,[2] and NaOH, diphenylamine reagent[5] was added and the tubes were heated at 100° C. for 30 minutes, cooled, and read at 620 mμ in the spectrophotometer. A small percentage of yeast-labile material (< 5 per cent of the total) was found in some of the inulin samples. No correction was made for this. Standards for both methods consisted of 3 dilutions of a sample of the infused inulin. The recovery of inulin added to plasma at the same 3 concentrations was determined. These concentrations correspond to 5, 10, and 15 mg. per cent

[1] Inulin was obtained from the Warner-Chilcott Laboratories. According to the manufacturers it contains less than 0.5 per cent fructose.

[2] One hundred gm. $ZnSO_4 \cdot 7H_2O$ and 40 ml. 6.25N H_2SO_4 diluted to 1,000 ml.

[3] The use of filter paper was found both to absorb inulin and add to the reagent blank, leading to spurious results.

[4] Since completion of these studies, it has been found that an 8 to 10 min. period of heating with alkali is adequate.

[5] Fourteen gm. diphenylamine (Mathesen), 600 ml. glacial acetic acid, 360 ml. conc. HCl.

[6] A constant temperature bath filled with oil and mechanically stirred provided better temperature regulation than a boiling water bath.

TABLE I

The ratio of the clearance of alkali-stable inulin to total inulin *

Subject	Age	Diagnosis	Average GFR *ml./min.*	Number of periods	Mean ratio	% Stable inulin
Ree	22	Normal	94	8	0.99	48
Hen	42	Renal artery thrombosis	32	2	1.00	52
Haz	29	Chronic glomerulonephritis	132	3	1.07	52
Gib	9	Addison's disease	54	4	1.06	52
Gib	9	Addison's disease	50	3	1.02	53
Gar	33	Nephrotic syndrome	82	3	0.97	50
Hol	39	Nephrotic syndrome	115	3	1.04	50
Win	50	Nephrotic syndrome	72	3	1.04	51
Win	50	Nephrotic syndrome	119	4	1.00†	56
Son	29	Nephrotic syndrome	25	4	0.99	51
Son	29	Nephrotic syndrome	47	5	1.01‡	55
Dot	9	Nephrogenic diabetes insipidus	57	7	0.96	58
Dot	9	Nephrogenic diabetes insipidus	45	7	1.05	53
Dot	9	Nephrogenic diabetes insipidus	35	7	0.96	51
Dot	9	Nephrogenic diabetes insipidus	46	6	0.99	51
Dot	9	Nephrogenic diabetes insipidus	55	2	0.99	53
Dot	9	Nephrogenic diabetes insipidus	42	8	0.95	48
				79		

* Mean of all observed periods, considering the repeated analyses in "Win" and "Son" as additional periods = 1.003 ± 0.011 (S.E.).
† Calculated from mean of three independent analyses by each method.
‡ Calculated from mean of five independent analyses by each method.

alkali-stable inulin in plasma in the alkali method and 10, 20, and 30 mg. per cent inulin in plasma in the yeast method. The fraction of the infused inulin resistant to alkali digestion was also determined in each instance. Alkali did not interfere with the color development.

In the later experiments the results were expressed in terms of a standard inulin solution consisting of pure alkali-stable inulin, prepared as follows:

Preparation of alkali-stable inulin. Fifty gm. of "Difco" inulin (approximately 85 per cent alkali-stable) were dissolved in 50 ml. of 1 N NaOH at 100° C. and heated for 2 hours. The solution was cooled under the tap and then placed in a cold room for several hours. One-hundred ml. of distilled water were added to the fudge-like mass and the precipitate suspended by prolonged stirring. Another 100 ml. of distilled water were added and the solution centrifuged for 30 minutes. The supernatant was decanted and the precipitate washed repeatedly in this manner, using progressively smaller volumes of water, until no further whitening occurred. Inulin was reprecipitated by heating at 100° C. and adding just enough water for solution, filtering, cooling rapidly, adding (with shaking) 2 volumes of acetone and filtering by suction. Twenty grams of white material were obtained. Inulin so prepared was not destroyed by yeast.

Analytical results. The relation between optical density and concentration is linear in both the yeast and the alkali methods, throughout the useful range of the colorimeter. The recovery of inulin added to plasma was 100.0 ± 2.3 [7] per cent in 67 samples analyzed by the alkali method and 100.7 ± 2.0 per cent in 62 samples analyzed by the yeast method. The plasma inuloid blank averaged 0.9 mg. per cent in the yeast method and 0.4 mg. per cent in the alkali method.

Additional observations on the alkali method. Using the procedure described, the range of 30 to 70 per cent transmission in the colorimeter corresponds to a range of 6 to 16 mg. per cent alkali-stable inulin in plasma. Analysis of a solution containing 500 mg. per cent glucose resulted in color equivalent to 0.3 mg. per cent alkali-stable inulin.[8] Autoöxidation of inulin was complete as shown by the fact that neither prolongation of the heating period, substitution of 7N for 4N alkali, nor continuous aeration of the tubes during heating with alkali altered the results.

RESULTS

Nine subjects were studied on 17 occasions with varying numbers of periods in each study, as shown in Table I. In addition, repeated analyses were performed on the samples from two of the studies. The original data from two representative experiments are shown in Table II together with the ratios of the clearance of alkali-stable (Ca) to "total" inulin as determined after yeasting (Cy). The ratios obtained from repeated analyses of the samples from two experiments are shown in Table III.

[7] Standard deviation.

[8] This ratio of inulin sensitivity to glucose sensitivity of about 1600:1 is 5 to 10 times greater than the ratio observed by Little (4).

TABLE II

Alkali-stable inulin clearance (Ca) and yeasted inulin clearance (Cy) in two representative experiments

Subject	Ca ml./min.	Cy ml./min.	Ca/Cy	Subject	Ca ml./min.	Cy ml./min.	Ca/Cy
	98.2	100.0	0.98		40.3	42.6	0.95
	98.2	96.2	1.02		47.0	51.9	0.91
	98.8	97.8	1.01		40.6	42.4	0.96
"Ree"	94.6	95.7	0.99	"Dot"	36.6	37.8	0.97
	94.1	98.4	0.96		42.7	43.9	0.97
	93.3	99.7	0.94		43.0	43.3	0.99
	94.9	97.5	0.97		38.5	40.2	0.96
	88.3	87.2	1.01		35.4	38.7	0.92

The mean of all the observed ratios of alkali-stable inulin clearance to "total" inulin clearance is 1.003, with a standard error of 0.011.

Several components of error can be discerned in these data. The standard deviation of the observed ratios obtained within each study is approximately 0.04. Variability of similar magnitude is found between the ratios from repeated analyses of the samples from a single study (Table III). The variability from subject to subject and from study to study within a single subject ("Dot") is only slightly greater. Considering the 17 studies in all, the standard deviation of a single ratio is 0.05. The variance of the mean was calculated as a suitable combination of the variance from study to study and the variance within a single study.[9] We may conclude that the average value of the ratio is between 0.983 and 1.023 at the 95 per cent level of confidence.

Since the inulin used was approximately one half alkali-stable and one half alkali-labile it follows that the clearance of alkali-stable inulin is on the average equal to that of alkali-labile inulin within approximately 4 per cent, at the 95 per cent level of confidence.

In view of the equality of the clearances of the two major fractions of inulin, it is of little importance whether the inulin used for measurement of filtration rate contains a large or small proportion of alkali-stable material. However, the analysis of inulin by means of the diphenylamine reaction after alkali treatment has several advantages over other methods: (a) The plasma inuloid blank is low; (b) the method is almost completely insensitive to glucose; and (c) low concentrations of inulin may be determined accurately.

Since alkali-labile inulin is destroyed in this analytical method, it is advantageous to employ inulin containing as much alkali-stable material

TABLE III

Ratios of alkali-stable to total inulin clearance determined from repeated analyses of the samples from two experiments

Subject	Period	Day of analysis 1	2	3	4	5
	1	1.05	1.08	1.01	1.06	1.00
	2	1.06	1.09	1.14	1.01	1.05
"Son"	3	1.01	1.04	0.94	0.95	0.98
	4	0.96	0.98	1.01	0.95	1.05
	5	1.04	1.01	0.96	0.98	1.03
	1	1.10	0.97	1.00		
"Win"	2	1.00	1.00	0.98		
	3	1.00	0.91	1.00		
	4	1.04	0.99	1.03		

[9] Note on Computation of the Variance of the Mean.

Computation of the variance of the mean was complicated by the fact that different numbers of periods were used in different studies and that the individuals had different numbers of studies. Thus, to assess the variability of the mean it was assumed that a given ratio was subject to a variance σ^2, attributable to study-to-study variation and also to a variance σ_e^2, attributable to technical error and period-to-period variability. This then implies that the mean for a given study has a variance $\sigma^2 + \frac{\sigma_e^2}{n_s}$, where n_s is the number of periods in the study. From this point of view, the mean as computed (a simple average) is equivalent to $\sum_{i=1}^{17} \frac{n_i \bar{x}_i}{n}$ where n_i is the number of periods in the "i"th study, $\bar{x}_i$ is the average ratio for the "i"th study and $n = \sum_{i=1}^{17} n_i$. From the above one can show that the variance of $\Sigma \frac{n_i \bar{x}_i}{n}$ is given by $\sigma^2 \frac{\sum_{i=1}^{17} n_i^2}{n^2} + \frac{\sigma_e^2}{n}$. The component σ_e^2 was estimated by the variance within studies averaged over all studies. To estimate σ^2 the sum of squares of deviations, $\sum_{i=1}^{17} n_i(\bar{x}_i - \bar{x})^2$, was first computed. Here $\bar{x}$ is the grand mean and other symbols are as previously defined. One can show that this sum of squares estimates $\left(n - \frac{\Sigma n_i^2}{n}\right)\sigma^2 + 16\,\sigma_e^2$. Consequently, this latter expression with our estimated value of σ_e^2 substituted was equated to the sum of squares. This equation was then solved for σ^2. The variance of $\bar{x}$ was estimated using the values so obtained for σ^2 and σ_e^2.

It should be remarked that taking the individual as a unit, it was found that the variance among individuals was of order comparable to the variance among studies. A variance of the mean computed on this basis was also comparable to the estimate obtained using studies as a unit.

as possible. The method of preparation described above is simpler than that described by Weil (9) and permits larger yields of pure alkali-stable material. However, inulin so prepared has only been used in animals in this laboratory. The present data do not establish that inulin purified after alkali treatment has the same clearance as the alkali-stable fraction of inulin prepared without alkali treatment.

SUMMARY

Improved methods are presented for the determination of alkali-stable inulin in plasma and urine and for the preparation of alkali-stable inulin. The renal clearance of the fraction of commercial inulin not destroyed by alkali is equal to the renal clearance of the fraction of inulin destroyed, within analytical error.

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HEMODYNAMICS AT REST AND DURING EXERCISE IN NORMAL PREGNANCY AS STUDIED BY CARDIAC CATHETERIZATION [1, 2]

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The physiological changes which take place during pregnancy are among the most extreme alterations occurring in non-pathological states. In addition to the welter of hormonal and body water changes, modifications in circulatory hemodynamics take place. Prior to the advent of cardiac catheterization, gas methods were utilized for the study of cardiac output in pregnant women (1–7). Data obtained in this manner indicated that the cardiac output increased during gestation. In general, the limitations of the gas methods for determination of cardiac output (8), *i.e.*, their relative inaccuracy, particularly in application to exercise studies, and the inability to obtain simultaneous measurements of pressures in the right side of the heart and pulmonary artery, led to several investigations of the cardiac hemodynamics using the catheter technique (9–13).

In the studies of Palmer and Walker (9) and Hamilton (10), the catheter was placed without radiographic or fluoroscopic control into what was believed to be the right atrium. The blood obtained from the catheter was assumed to be mixed venous blood and pressures were taken with a saline manometer. Values for arterial oxygen content were assumed, thus making the A–V oxygen difference an approximation. Werkö, Bucht, Lagerlöf, and Holmgren's (11, 13) careful studies did not include any observations on normal pregnant women in the mid-trimester.

No data obtained by adequate catheterization techniques are available concerning the cardiac output and right heart pressures in normal pregnant women throughout gestation. No studies have reported the cardiac hemodynamic response to exercise in these subjects. It is the purpose of this paper to present such information.

MATERIAL AND METHODS

Cardiac catheterization was performed on 46 normal pregnant women selected from the pre-natal clinic of The Mount Sinai Hospital. History, physical examination, electrocardiogram, chest roentgenogram and fluoroscopy revealed no evidence of organic heart disease. Each patient was studied once during gestation, from the fourteenth week to one day prior to delivery at term.

The patients were divided into the following groups: Group I, 14 to 24 weeks of gestation (8 patients); Group II, 25 to 27 weeks (8 patients); Group III, 28 to 30 weeks (11 patients); Group IV, 31 to 35 weeks (9 patients); and Group V, 36 to 40 weeks (11 patients). No studies were performed prior to the 14th week of gestation because of the possible deleterious effects of radiation on the fetus.

With the patient in a basal, post-absorptive state, without pre-medication, catheterization of the right heart and pulmonary artery was carried out. Special care was taken to shield the patient and fetus from excess radiation. An indwelling needle was placed in the brachial artery. Pressures were recorded from the right atrium and ventricle, pulmonary artery and brachial artery and, in about one-half the cases, from the pulmonary wedge position by means of strain gauge manometers (Statham No. p 23 A) using a four channel cathode ray oscilloscopic photographic recording system.[6] Cardiac output was determined by the Fick principle. Blood collected simultaneously from the pulmonary and brachial arteries was analyzed for oxygen content and capacity by the method of Van Slyke and Neill (14) with a maximum permissible difference between duplicate analyses of 0.2 volume per cent. Oxygen consumption was measured employing the closed system in 14 patients (Benedict Roth spirometer) and the open system in 32 patients. Expired gas was analyzed by micro Scholander tech-

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[6] Manufactured by Electronics for Medicine, Inc., New York, N. Y.

TABLE I

14–24 Weeks

SUBJ.		WEEK OF PREG.	B.S.A.	AGE	$\dot{V}O_2$ cc/min. M^2	ARTERIAL BLOOD O_2 CONT. VOL.%	O_2 SAT.%	C.O.	C.I.	A-V Diff. VOL.%	H.R.	S.V. cc.	Δ C.O. / Δ VO_2	BRACHIAL ARTERY S.	D.	M.	"P.C."	PULMONARY ARTERY S.	D.	M.	RIGHT VENT. S.	D.	T.P.R.
C.T.	R	14	1.56	38	117	13.7	97	5.05	3.27	3.6	90	56	748	--	--	--	--	13	2	6	15	1	--
	E				221	13.8	96	6.27	4.02	5.5	100	63		--	--	--	--	20	7	11	20	0	--
A.M.	R	18	1.54	24	145	14.0	97	6.76	4.40	3.3	88	77	870	117	68	90	--	17	7	12	19	5	1003
	E				185	13.8	93	7.30	4.78	3.9	96	77		120	74	92	--	18	5	12	20	3	975
M.C.	R	18	1.64	25	130	13.8	98	7.01	4.28	3.0	85	83	--	95	53	72	--	18	7	11	17	4	765
	E				--	--	--	--	--	--	--	--		--	--	--	--	22	7	12	22	3	--
J.M.	R	19	1.79	31	144	14.6	97	7.14	3.99	3.6	94	76	--	107	68	81	--	21	8	13	21	4	863
	E				--	--	--	--	--	--	--	--		106	70	81	--	23	9	13	23	5	--
D.C.	R	22	1.59	22	129	14.4	99	6.82	3.79	3.4	102	67	860	--	--	--	--	15	3	7	16	0	--
	E				279	14.1	96	8.88	5.58	5.0	125	71		157	84	104	--	15	7	10	17	2	900
E.G.	R	23	1.60	19	136	14.6	98	6.81	4.23	3.2	108	63	2860	146	83	107	6	19-	7	11	17	0	1220
	E				232	14.6	95	8.43	5.24	4.4	112	75		153	83	103	--	19	9	12	19	3	948
C.M.	R	23	1.46	15	147	11.0	96	6.51	4.49	3.3	93	70	1928	117	67	80	--	--	--	--	20	4	1180
	E				232	10.9	96	8.88	6.12	3.8	111	80		122	79	90	--	--	--	--	21	4	794
A.V.	R	24	1.44	29	154	13.4	98	6.14	4.26	3.6	90	68	--	111	56	71	3	18	8	12	18	3	885
	E				--	--	--	--	--	--	--	--		100	56	--	--	19	8	14	--	--	
Mean R			1.58	25.4	138			6.53	4.09	3.4	99	70											986
S.D.					±12			±0.21	±0.39	±0.2	±8	±9											±183
Mean E					230			7.94	5.15	4.5	109	73	1093										904
S.D.					±34			±1.09	±0.80	±0.2	±11	±7	±480										

PRESSURES IN mm. Hg

B.S.A. - - - - - - - - - Body surface area in meter^2
VO_2 - - - - - - - - - Oxygen consumption
C.O. - - - - - - - - - Cardiac output in liters per minute
C.I. - - - - - - - - - Cardiac index in liters per minute per square meter
A-V Diff. - - - - - - - Arterio-venous oxygen difference
H.R. - - - - - - - - - Heart rate per minute
S.V. - - - - - - - - - Stroke volume
$\frac{\Delta \text{ CO}}{\Delta VO_2}$ - - - - - - - - - Increase in cardiac output in cc per 100 cc increase in O_2 consumption during exercise
T.P.R. - - - - - - - - - Total peripheral resistance in dynes seconds centimeters-5
R - - - - - - - - - Rest
E - - - - - - - - - Exercise
S.D. - - - - - - - - - Standard deviation
"P.C." - - - - - - - - - Mean pulmonary capillary wedge pressure

TABLE II

25–27 Weeks

SUBJ.		WEEK OF PREG.	B.S.A.	AGE	$\dot{V}O_2$ cc/min. M^2	ARTERIAL BLOOD O_2 CONT. VOL.%	O_2 SAT.%	C.O.	C.I.	A-V Diff. VOL.%	H.R.	S.V. cc.	A C.O. $\dot{V}O_2$	PRESSURES IN mm. Hg BRACHIAL ARTERY S.	D.	M.	"P.C."	PULMONARY ARTERY S.	D.	M.	RIGHT VENT. S.	D.	T. P. R.
G.P.	R	25	1.48	29	139	15.5	97	6.84	4.64	2.85	90	75	778	107	61	85	6	19	7	10	--	--	---
	E				290	15.8	98	8.58	5.80	5.0	115	75		122	73	98	--	19	5	10	16	3	885
V.B.	R	26	1.46	22	160	15.0	98	6.48	4.44	3.6	91	71	1446	109	66	80	11	24	8	16	24	7	980
	E				243	14.5	97	8.23	5.64	4.3	100	82		106	65	83	--	22	5	16	23	5	756
R.M.	R	26	1.78	18	124	13.0	96	4.70	2.64	4.7	100	47	739	92	62	69	2	15	4	7	15	2	1430
	E				191	13.2	99	5.58	3.13	6.1	115	49		97	56	65	--	13	5	8	14	1	903
N.M.	R	26	1.53	24	146	14.0	99	7.42	4.82	3.0	95	78	1390	125	65	85	6	22	8	13	25	5	895
	E				203	13.8	98	8.64	5.61	3.6	110	79		110	68	86	--	28	4	18	25	3	778
C.P.	R	27	1.82	24	160	14.3	98	8.34	4.58	3.5	98	85	1127	--	--	--	--	25	8	11	27	3	---
	E				221	14.3	97	9.58	5.26	4.2	115	83		133	83	102	--	29	16	19	26	6	820
R.H.	R	27	1.86	18	129	11.8	97	8.00	4.30	3.0	92	87	---	114	72	89	--	12	7	10	15	3	860
	E				--	--	--	--	--	--	--	--		131	76	102	--	12	3	7	17	3	---
M.T.	R	27	1.58	22	130	13.7	97	7.76	4.91	2.65	83	94	954	113	67	88	5	21	7	13	22	3	895
	E				268	14.1	99	9.84	6.20	4.30	100	98		107	52	66	--	25	9	15	24	8	487
A.U.	R	27	1.63	28	160	15.6	98	6.19	3.78	4.1	94	66	582	103	64	83	1	15	5	8	15	2	1078
	E				242	15.8	97	6.94	4.26	5.5	102	68		119	81	99	--	17	7	10	20	10	1063
Mean	R		1.64	23.1	143			6.96	4.26	3.4	93	75											1089
S.D.					±15			±1.19	±0.74	±0.2	±5	±15											±220
Mean	E				237			8.20	5.13	4.7	108	76	1002										813
S.D.					±35			±1.48	±1.05	±0.3	±7	±15	±332										±176

TABLE III

28–30 Weeks

SUBJ.		PREG.	B.S.A.	AGE	$\dot{V}O_2$ cc/min. M^2	ARTERIAL BLOOD O_2 CONT. VOL.%	O_2 SAT.%	C.O.	A-V diff. C.I.	A-V diff. VOL.%	H.R.	S.V. cc.	$\frac{\Delta C.O.}{\Delta \dot{V}O_2}$	PRESSURES IN mm. Hg BRACHIAL ARTERY S.	D.	M.	"P.C."	PULMONARY ARTERY S.	D.	M.	RIGHT VENT. S.	D.	T. P. R.
A.D.	R	26	1.66	25	155	11.3	100	8.30	5.00	3.1	107	77		112	70	90	--	--	--	--	26	7	800
	E				242	11.1	99	9.94	5.99	4.0	120	83	1130	---	--	--	--	--	--	--	27	7	---
G.R.	R	28	1.92	34	137	15.2	98	6.90	3.60	3.8	96	72		136	77	99	--	21	10	14	15	0	1110
	E				206	15.0	96	8.22	4.26	4.8	114	72	1000	---	--	--	--	21	13	16	23	7	---
M.L.	R	28	1.47	18	115	13.4	100	4.15	2.81	4.1	85	49		112	77	89	7	19	8	13	20	5	1660
	E				227	14.2	101	5.48	3.75	6.1	100	55	806	140	92	110	--	21	8	13	23	5	1590
C.M.	R	30	1.78	18	120	13.1	96	5.78	3.25	3.7	92	63		118	65	83	--	17	4	10	16	1	1130
	E				263	13.5	94	7.95	4.45	5.9	118	68	869	114	76	89	--	26	5	11	20	2	875
E.B.	R	30	1.80	19	122	14.3	98	6.73	3.74	3.3	79	85		---	--	--	--	20	8	12	20	4	---
	E				205	14.2	96	8.04	4.46	4.6	95	85	885	123	75	94	--	28	16	20	25	7	865
O.Y.	R	30	1.53	21	176	15.4	97	6.12	4.00	4.4	105	58		128	77	93	3	20	8	12	19	1	1212
	E				---	---	---	---	---	---	---	--	---	---	--	--	--	16	6	9	18	2	---
E.C.	R	30	1.75	33	161	14.6	97	7.62	4.35	3.7	100	76		132	82	97	8	20	5	10	22	5	976
	E				---	---	---	---	---	---	---	--	---	146	89	107	--	18	6	10	21	1	---
A.L.	R	30	1.59	26	137	13.6	99	6.60	4.16	3.3	100	66		95	59	73	4	16	6	11	16	3	850
	E				246	13.9	100	7.67	4.83	5.1	115	67	618	111	68	87	--	27	13	18	27	4	875
M.P.	R	30	1.50	22	135	13.1	97	6.96	4.65	2.9	100	70		110	64	80	1	13	3	8	14	3	885
	E				269	13.0	96	8.24	5.52	4.9	107	77	633	103	63	80	--	14	5	8	14	0	775
G.D.	R	30	1.79	28	113	12.9	95	6.73	3.76	3.0	80	84		--	--	--	--	22	11	15	16	3	---
	E				220	13.0	95	8.20	4.59	4.8	96	85	765	—	--	--	--	--	--	--	20	7	---
Mean	R		1.68	25.4	137			6.59	3.93	3.5	94	70											1078
S.D.					± 21			± 1.08	±0.65	±0.2	±10	±11											±277
Mean	E				236			7.97	4.73	5.0	108	74	838										996
S.D.					± 24			± 1.21	±0.71	±0.2	±10	±11	±173										±335

TABLE IV

31–35 Weeks

SUBJ.		WEEK OF PREG.	B.S.A.	AGE	$\dot{V}O_2$ cc/min. / M²	ARTERIAL BLOOD O_2 CONT. VOL.%	ARTERIAL BLOOD O_2 SAT.%	C.O.	C.I.	A-V Diff. VOL.%	H.R.	S.V. cc.	Δ C.O. / Δ VO_2	PRESSURES IN mm. Hg: BRACHIAL ARTERY S.	BRACHIAL ARTERY D.	BRACHIAL ARTERY M.	"P.C."	PULMONARY ARTERY S.	PULMONARY ARTERY D.	PULMONARY ARTERY M.	RIGHT VENT. S.	RIGHT VENT. D.	T.P.R.
D.C.	R	31	1.60	23	140	11.7	95	5.47	3.43	4.1	100	55		---	--	--	--	16	8	10	14	2	---
	E				---	---	--	---	---	--	---	--	---	---	--	--	--	--	-	--	--	-	---
D.H.	R	31	1.78	22	137	13.6	96	4.96	2.87	4.9	85	58		137	80	93	--	22	4	9	20	2	1468
	E				231	13.7	95	6.98	3.94	5.9	100	70	1196	---	--	--	--	21	5	13	23	4	---
E.B.	R	32	1.92	19	132	12.6	95	6.68	3.48	3.8	83	80		115	67	84	--	15	3	8	15	3	970
	E				295	12.8	95	9.31	4.85	6.1	111	84	840	118	70	86	--	15	5	9	17	2	721
A.P.	R	32	1.58	21	135	13.0	95	5.94	3.91	3.6	75	79		96	56	68	7	13	4	8	17	4	864
	E				208	12.9	94	7.63	5.02	4.3	100	76	1483	---	--	--	--	18	5	10	18	1	---
L.B.	R	32	1.48	21	169	13.9	96	6.94	4.69	3.6	94	74		119	68	92	8	21	8	13	23	6	991
	E				300	13.9	95	9.47	6.40	4.7	115	82	1298	146	89	107	--	23	9	15	24	7	880
M.C.	R	33	1.54	24	130	16.4	96	5.26	3.42	3.8	64	82		124	73	98	--	22	8	14	23	7	1395
	E				---	---	--	---	---	--	---	--	---	138	87	106	--	20	8	14	22	4	---
J.R.	R	34	1.68	33	132	13.7	96	4.86	2.98	4.6	88	55		---	--	--	--	17	8	11	18	3	---
	E				---	---	--	---	---	--	---	--	---	---	--	--	--	17	8	11	18	2	---
C.A.	R	34	1.51	23	157	15.1	96	6.08	4.00	3.9	100	61		---	--	--	5	22	9	15	23	10	---
	E				276	14.9	94	9.06	6.00	4.6	115	79	1660	---	--	--	--	20	9	14	24	10	---
A.V.	R	34	1.54	21	145	13.5	97	5.60	3.64	4.0	100	56		---	--	--	6	17	6	11	17	1	---
	E				189	13.1	96	6.22	4.04	4.7	104	60	910	---	--	--	--	21	11	15	22	6	---
Mean	R		1.63	23.0	142			5.75	3.60	4.0	88	67											1138
S.D.					±13			±0.72	±0.55	±0.1	±13	±12											± 274
Mean	E				250			8.11	5.04	5.1	108	75	1231										800
S.D.					±47			±1.43	±1.00	±0.2	±7	±9	±319										

TABLE V
36–40 Weeks

SUBJ.		WEEK OF PREG.	B.S.A.	AGE	$\dot{V}O_2$ cc/min. M^2	ARTERIAL BLOOD O_2 CONT. VOL.%	ARTERIAL BLOOD O_2 SAT.%	C.O.	C.I.	A-V Diff. VOL.%	H.R.	S.V. cc.	Δ C.O. / ΔVO_2	PRESSURES IN mm. Hg BRACHIAL ARTERY S.	D.	M.	"P.C."	PULMONARY ARTERY S.	D.	M.	RIGHT VENT. S.	D.	T.P.R.
C.W.	R	36	1.59	21	164	15.3	99	5.20	3.27	5.0	95	55		114	71	89	--	20	11	15	17	2	1310
	E				298	15.2	99	6.67	4.20	7.1	117	57	732	116	72	88	--	18	8	14	23	4	1020
N.M.	R	36	1.49	23	146	13.1	99	4.19	2.81	5.2	100	42		---	--	--	5	14	5	11	11	3	---
	E				203	12.4	95	5.30	3.56	5.7	115	46	1320	---	--	--	--	17	8	11	18	3	---
M.M.	R	37	1.67	21	156	11.9	96	5.93	3.55	4.4	100	59		102	74	87	3	12	3	8	15	2	1145
	E				280	12.3	94	7.67	4.59	6.1	105	73	840	133	75	96	--	13	3	7	13	1	1000
D.C.	R	37	1.55	17	154	14.5	97	4.85	3.13	4.9	96	51		100	66	80	--	--	-	--	19	2	1288
	E				224	14.6	97	6.20	4.00	5.6	118	53	1240	125	78	90	--	--	-	--	21	3	1125
M.M.	R	37	1.53	17	154	11.9	98	6.38	4.17	3.7	96	66		128	72	93	9	19	10	13	22	4	1135
	E				---	---	--	---	---	--	---	--	---	140	88	109	--	--	-	--	27	3	---
V.S.	R	38	1.49	22	149	14.8	99	5.16	3.44	4.3	98	53		---	--	--	5	14	2	6	17	1	---
	E				245	14.7	97	6.65	4.43	5.5	115	58	1350	---	--	--	--	18	3	7	18	0	---
M.Mu.	R	38	1.59	23	144	15.5	97	5.70	3.59	4.0	80	71		---	--	--	4	16	5	9	--	-	---
	E				238	15.6	97	7.28	4.57	5.2	91	80	1055	---	--	--	--	20	6	10	24	6	---
A.P.	R	39	1.61	27	133	15.4	97	4.97	3.09	4.3	91	55		115	74	89	4	19	6	13	20	3	1430
	E				236	15.4	94	7.76	4.82	4.9	108	72	1680	122	82	104	--	15	5	10	--	-	1070
O.C.	R	39	1.56	22	148	15.2	96	6.58	4.23	3.5	108	61		105	65	81	7	18	7	11	20	3	975
	E				---	---	--	---	---	--	---	--	---	118	79	92	--	18	6	11	20	4	---
O.B.	R	40	1.68	29	135	15.2	98	5.79	3.45	3.8	92	63		120	75	93	--	20	9	14	--	-	1240
	E				210	14.7	98	6.84	4.08	5.0	100	68	860	137	80	106	--	21	6	14	20	4	1205
M.K.	R	40	1.94	23	164	16.3	99	6.12	3.16	5.2	100	61		145	97	111	--	18	5	11	19	0	1410
	E				272	16.1	98	7.75	4.00	6.8	115	65	776	150	96	112	--	30	15	20	23	1	1148
Mean	R		1.61	22.3	150			5.53	3.44	4.4	96	58											
S.D.					± 10			± 0.72	± 0.44	± 0.2	± 7	± 8											1244
Mean	E				245			6.90	4.25	5.8	110	64											± 152
S.D.					± 32			± 0.82	± 0.39	± 0.2	± 9	± 11	1095 ± 323										1095 ± 79

TABLE VI

Statistical data

Comparison of groups for probability of chance occurrence of difference (Groups compared)

		I-II	I-III	I-IV	I-V	II-III	II-IV	II-V	III-IV	III-V	IV-V
VO2/M2 c.c./min.	R				< .05						< .10
	E										
Cardiac Index l./min.	R			< .10	< .01		< .10	< .02		< .10	
	E				.05			< .10			< .10
A-V O2 Diff. Vol. %	R			< .01	< .01		.05	< .01		.01	< .10
	E										
Total Peripheral Resistance	R				< .10			.05		< .10	

All blank spaces represent values for $P > .10$
R----Rest
E----Exercise

nique (15) with a maximum permissible difference between duplicate analysis of 0.04 volume per cent.

Two control determinations of cardiac output were made at rest and this measurement repeated after ten minutes of exercise which consisted of pedaling a stationary bicycle in the recumbent position at a steady rate. In those instances in which the open system was used, the data presented meet the criteria for the steady state (16). Where the closed system was employed, duplicate control oxygen consumptions were required to agree within 10 per cent. Pulmonary artery pressures were measured three or four times at rest, and at 3, 6, 9, and 12 minutes of exercise. Right atrial and ventricular pressures were obtained at rest and after 12 minutes of exercise. Similar control measurements of brachial artery pressure were made at rest and repeated during exercise.

Average figures for control cardiac output and pulmonary artery pressure values are presented. Peripheral resistance was calculated according to the following formula:

$$\text{Peripheral Resistance (dyne sec. cm.}^{-5}) = \frac{1{,}332\ (\text{mean brachial artery pressure} - \text{mean rt. atrial pressure mm. Hg})}{\text{cardiac output (ml./sec.)}}$$

Statistical analysis was carried out by the non-parametric test of unpaired replicates (16A). Standard deviation was calculated separately.

RESULTS

Results including standard deviation of the mean values are presented in Tables I to V. Comparison of the individual groups statistically is presented in Table VI.

Oxygen consumption

During the period of gestation studied, average oxygen consumption was found to be slightly and consistently elevated, reaching a value of 9 per cent greater than normal at term. The calculations were based on the body surface area from the standard Du Bois formula (17). Although it is recognized that this formula may not be entirely valid for the pregnant female, it is believed that the presentation of the data in terms of body surface area offers a more meaningful basis of comparison of the various groups than absolute values.[7] The increments in oxygen consumption during exercise, while differing slightly in the various groups, were in general comparable (Tables I to VI, Figure 1).

Arterio-venous oxygen difference

There is a considerable reduction in the average A-V oxygen difference (Figure 2) at rest in Groups I, II and III (14 to 30 weeks) with a gradual return towards normal in the last ten weeks of gestation. In Group I (14 to 24 weeks), the average A-V oxygen difference at rest was only 3.4 volumes per cent. It remained at approximately this level in Groups II and III, then increased in Group IV (31 to 35 weeks) to 4.0 volumes per cent. In Group V (36 to 40 weeks), it reached its highest level: 4.4 volumes per cent,

[7] The Du Bois Formula for body surface area is

$$\text{Area (cm.}^2) = \text{wt.}^{0.425} \times \text{ht.}^{0.725} \times 71.84.$$

It is apparent that changes in weight alone as take place in pregnancy influence surface area to a lesser extent than height. The use of data reduced to square meter of body surface area is essential, since serial studies on the same patient could not be carried out, and comparisons of groups of patients studied at various stages of pregnancy were therefore necessary.

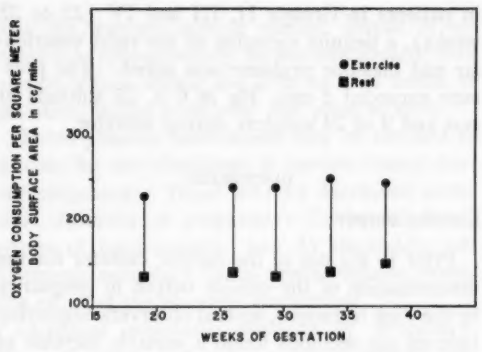

FIGURE 1

a normal value. During exercise, a variable increase in the average A-V oxygen difference occurred, but the absolute level of A-V oxygen difference was somewhat lower in Groups I to IV as compared with the patients studied just prior to term.

Cardiac output

It follows from the data presented above that the average resting cardiac output was elevated in Groups I, II and III, and reached its peak during the 25th to 27th weeks of gestation. Later in pregnancy (Groups IV and V), the average resting cardiac output fell toward normal, reaching its lowest level at the 36th to 40th weeks (Figure 3). During exercise, the increase in cardiac output per hundred cubic centimeter increment in oxygen consumption was within established normal limits (18), in all but one case which was only slightly below the lower limit of normal (Figure 4).

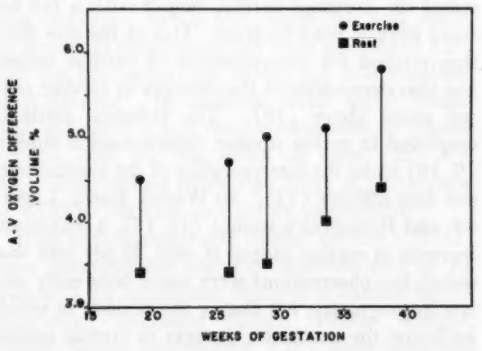

FIGURE 2

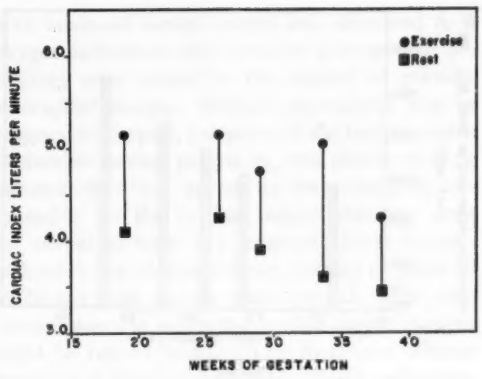

FIGURE 3

Peripheral resistance

No remarkable change in the systemic arterial pressure was noted during the course of gestation. It follows, therefore, from the increase in cardiac output that a decrease in peripheral resistance must take place. During the 14th to 24th weeks of gestation, the lowest average values for peripheral resistance at rest were obtained (980 dyne sec. cm.$^{-5}$). As pregnancy advanced, the peripheral resistance rose progressively, reaching a normal value of 1240 dyne sec. cm.$^{-5}$ at term (Figure 5).

Pressures

Pulmonary arterial and right ventricular systolic pressures at rest were within normal limits. During exercise, a slight but definite elevation was noted in all five groups. Pulmonary arterial and

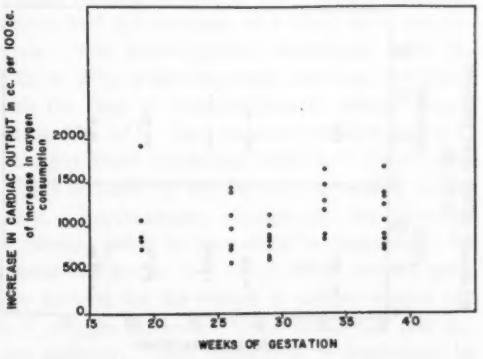

FIGURE 4

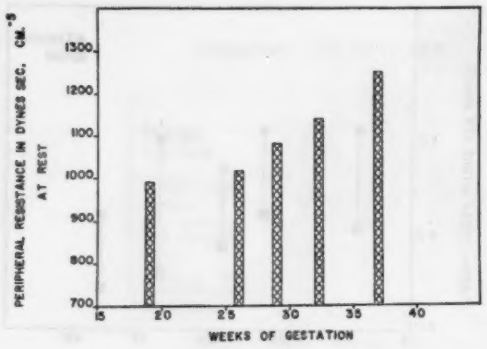

FIGURE 5

right ventricular systolic pressures during exercise did not exceed 30 mm. Hg in any subject. Pulmonary arterial diastolic pressure was within normal limits at rest and increased slightly during exercise, exceeding 12 mm. Hg in 5 subjects. Mean pressure in the pulmonary artery was within normal range (Figure 6). During exercise, it rose above 15 mm. Hg in 6 subjects, reaching a level of 20 mm. Hg in two; the remaining cases showed only a slight increment over resting values. "Pulmonary capillary" pressure was within normal limits. No remarkable trends were noted in right ventricular systolic, pulmonary arterial systolic, diastolic and mean, and "pulmonary capillary" pressures as pregnancy progressed.

The level of right ventricular end diastolic pressure, appears to be related to the period of gestation (Figure 7). In Groups I and V, the right ventricular end diastolic pressure was not above the upper limit of normal at rest. However,

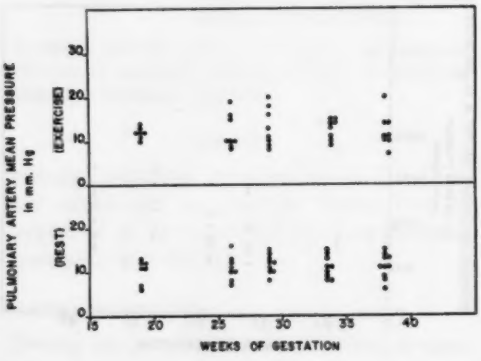

FIGURE 6

in patients in Groups II, III and IV (25 to 35 weeks), a definite elevation of the right ventricular end diastolic pressure was noted. The pressure exceeded 5 mm. Hg in 6 of 27 subjects at rest and 9 of 24 subjects during exercise.

DISCUSSION

Cardiac output

Prior to the use of the cardiac catheter for the determination of the cardiac output in pregnancy by the Fick technique, several observers employing indirect gas methods found a variable increase of 30 to 85 per cent in the resting cardiac output during gestation (1–7). Burwell, Strayhorn, Flickinger, Corlette, Bowerman, and Kennedy

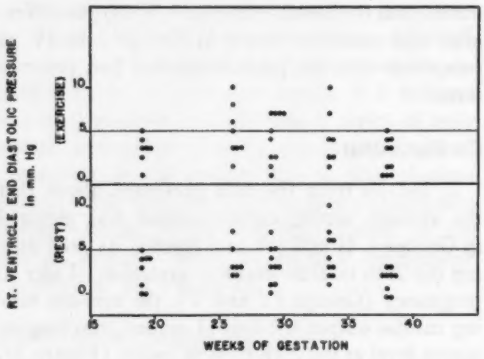

FIGURE 7

utilized the foreign gas technique and performed serial studies in 4 patients throughout gestation (7). While the gas techniques have their recognized limitations (8), these serial studies illustrated an increased cardiac output with a fall toward normal prior to term. Use of the dye dilution method for determination of cardiac output has also demonstrated the changes in cardiac output noted above (19). The technical methods employed in earlier cardiac catheterization studies (9, 10) make the interpretation of the cardiac output data difficult (13). In Werkö, Bucht, Lagerlöf, and Holmgren's studies (11, 13), a maximum increase in cardiac output of only 20 per cent was noted, but observations were made only early and late in pregnancy, not during the periods in which we found the maximum changes in cardiac output to take place. The present study demonstrates

that the average resting cardiac output is increased to approximately 40 per cent greater than normal values during the 25th to 27th weeks of gestation, falling to normal just prior to term.

Three possible mechanisms may be invoked to account for the alterations in cardiac output during pregnancy. These are: 1) Increased metabolic demands of pregnancy; 2) hemodynamic effects of hypervolemia; and 3) circulatory adjustments secondary to an arterio-venous shunt-like effect of the placental circulation.

Increased metabolic demands of pregnancy. Were the increased cardiac output related primarily to increased metabolic demands of pregnancy, this should be reflected in a proportional increase in maternal oxygen consumption. However, in contrast to the 40 per cent maximum increase in cardiac output, the average oxygen consumption was elevated less than 10 per cent. The latter figure is in general agreement (20) or only slightly lower than the findings reported by others (21). It may be concluded that increased metabolic demands of pregnancy, as reflected in the maternal oxygen consumption, cannot primarily account for the increased cardiac output. This view is further supported by the findings of a marked reduction in the maternal A-V oxygen differences associated with the elevated cardiac output.

Hemodynamic effects of hypervolemia. Numerous studies have confirmed the existence of significant hypervolemia during gestation (22–27). Thomson, Hirsheimer, Gibson, and Evans (24) reported a peak increase in plasma volume in the ninth lunar month of 65 per cent, falling to a value 50 per cent greater than normal just before delivery. While this slight fall was noted, its statistical significance has been questioned (26) and the pre-parturition values are still considerably elevated. In addition, other serial studies have indicated a progressive increment in plasma volume with the maximum at term (25, 27). Most observations agree that there is an increase of 40 to 50 per cent just before parturition.

Both experimental and clinical studies (28–33) have demonstrated that hypervolemia may be responsible for significant changes in cardiac hemodynamics. Recently, Eichna, Farber, Berger, Rader, Smith, and Albert (33) have described patients in whom hypervolemia was associated with increased cardiac output and decreased A-V oxygen difference and in whom a congested circulatory state existed in the absence of primary myocardial disease. While hypervolemia may be responsible, in part, for some of the hemodynamic alterations during pregnancy, the plasma volume increase does not appear to be principally responsible for the cardiac output changes, since the output at term has returned almost to normal, while the plasma volume remains elevated 40 to 50 per cent greater than normal. The only circumstances in which the cardiac output changes could be related to alterations in plasma volume would be if prior to term a significant redistribution of blood took place, with pooling of blood in the dependent venous beds and decreased venous return to the heart. Such a decrease has been reported to take place in the last month of gestation (13). At this time, elevation of venous pressure in the lower extremities without a concomitant rise in the upper extremities has also been reported (34). Finally, the absence of elevation of right ventricular end diastolic pressure in the last month of gestation (in contrast to the distinct elevation noted in some patients during the 25th to 35th week) would be consistent with a reduction in intrathoracic blood volume, with pooling elsewhere.

A-V shunt-like effect of placenta. Burwell (35) has presented the interesting hypothesis that the placenta may behave as a modified A-V fistula, which may account for the circulatory changes in pregnancy. This was based upon the increased heart rate, slight fall in systemic diastolic pressure, increased systemic pulse pressure, higher oxygen content of uterine vein blood in pregnant bitches and the presence of a bruit over the placenta. The hemodynamic alterations from the 14th to 27th weeks reported here are consistent with the type of changes seen in arterio-venous fistula (36, 37). The increased venous return to the right heart associated with A-V fistula may in part account for the increase in cardiac output noted. Furthermore, changes in the placental circulation prior to term may be responsible for a reduction in the A-V shunt effect and, in turn, may account for the return of cardiac output and A-V oxygen difference towards normal just before delivery. This hypothesis is supported by the physiological observations of Barcroft, Her-

kel, Hill, Flexner, McCarthy, and McClurkin (38, 39) who found that uterine vein oxygen content in the rabbit is relatively elevated until the 18th to 20th day of gestation (comparable to the 7th to 8th month in the human female) after which there is a reduction in uterine vein oxygen content. Morphological studies of the placenta not only have demonstrated A-V shunt-like apparatus (40, 41) but have also indicated that in the last weeks of gestation progressive senescence occurs, with obliteration of portions of the maternal placental circulation (42). Further evidence for aging of the placenta is found in studies of placental tissue respiration which have demonstrated a fall in the oxygen utilization by the placenta in the last month of gestation (43).

The alterations in cardiac output in normal pregnancy do not offer conclusive information concerning their mechanism.[8] However, from the considerations presented above, it would appear that while all three factors may play a role, either redistribution of blood, the senescence of a placental A-V shunt, or a combination of these factors can explain the fall in cardiac output towards normal at term. The concept of obliteration of portions of the placental circulation is supported by the findings of a decreased total peripheral resistance during the second trimester, with a progressive return towards normal at term.

Right heart and pulmonary vascular pressures

Elevation of pulmonary artery mean and diastolic pressure was observed in several patients during exercise, but the most noteworthy of the changes in pressure observed was the elevation of right ventricular end diastolic pressure in the 25th to 35th weeks. Three possibilities could be invoked to explain this: 1) Right heart failure; 2) intrapleural pressure changes; and 3) effects of hypervolemia.

Elevation of the right ventricular end diastolic pressure as measured by conventional catheter technique has been regarded as evidence of right ventricular failure. However, in our patients the presence of an increased cardiac output at rest associated with a markedly decreased A-V oxygen difference, and the findings of normal increase in cardiac output per unit increment in oxygen consumption on exercise, are not consistent with this possibility. The possibility exists that elevation of the intrapleural pressure during pregnancy may account for the measured elevations in right ventricular end diastolic and pulmonary artery mean and diastolic pressures. This could be related to elevation of the diaphragm, and the return of pressures to normal during the last week of gestation may be due to descent of the fetus and diaphragm which occurs frequently at this time. However, in the absence of direct measurement of intrapleural pressure, no conclusive statement is warranted. Finally, as previously noted, a congested circulatory state not related to myocardial failure (33) and associated with an increased venous return may be associated with elevations of pressure in the right heart and lesser circulation.

SUMMARY AND CONCLUSIONS

1. Cardiac hemodynamics was studied by cardiac catheterization in 46 normal pregnant women, from 14 weeks of gestation to term.

2. Arterio-venous oxygen difference was decreased to an average of 3.4 volume per cent during the 14th to 30th weeks, increasing toward normal in the month prior to term.

3. Average resting cardiac index was elevated early in pregnancy, reaching a maximum of 4.26 L. per minute at the 25th to 27th weeks, falling toward normal just prior to term.

4. Pressures in the brachial artery showed no significant abnormalities and calculated total peripheral resistance at rest was decreased from the 14th to 27th weeks, rising progressively to normal at term. Cardiac output increased normally on exercise in all but one patient.

5. Pressures measured in the right heart and pulmonary artery revealed only slight increases in pulmonary arterial pressures without significant elevations above normal. Right ventricular end

[8] In considering the possible role of the anemia of pregnancy on the cardiac output (44), it should be noted that in spite of the moderate reduction in hemoglobin, the total red cell mass in pregnancy is actually increased (25). In this study, the oxygen capacity averaged 14.7 volumes per cent. This moderate reduction in hemoglobin was practically uniform throughout gestation and no correlation with the degree of the anemia and level of cardiac output could be established.

diastolic pressure was elevated in 6 of 27 patients at rest in the 25th to 35th weeks.

6. The possible mechanisms for the cardiac output alterations include: (a) Increased metabolic demands of pregnancy; (b) hemodynamic effects of hypervolemia; and (c) A-V shunt-like effect of the placenta. The possible role of each of these factors has been discussed.

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CLINICAL AND PHYSIOLOGICAL ASPECTS OF A CASE OF OBESITY, POLYCYTHEMIA AND ALVEOLAR HYPOVENTILATION

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The occurrence of arterial hypoxia with polycythemia usually results from some known type of pulmonary disease or from an abnormal communication between the right and left sides of the circulation. Although arterial oxygen unsaturation has been observed in patients with polycythemia vera (1–4), it is usually of only mild degree. Recently, Newman, Feltman, and Devlin (5) found polycythemia and a severe degree of arterial hypoxia in two patients who did not have evidence of any previously described form of lung disease. These authors postulated that polycythemia vera by its effects on the lung or on the respiratory center could give rise to oxygen unsaturation of arterial blood. They also suggested that in some patients polycythemia is secondary to respiratory center disease of undetermined etiology.

It is the purpose of the present report to present the clinical and physiological findings in an obese young man with polycythemia and arterial oxygen unsaturation.

CLINICAL REPORT

C. D., a thirty-year old white man was admitted to the hospital on January 8, 1954 complaining of shortness of breath and ankle swelling. He had weighed more than 200 pounds for at least 13 years. In 1942 he worked as a furnace feeder in an aluminum plant for six months. He first noted a non-productive cough in 1950 and since then had experienced an increased frequency of respiratory infections. In March, 1952, an elevated hematocrit was observed, and by October, 1952 he had to stop work because of dyspnea and ankle edema. In the six months before entering the hospital these symptoms increased, and he developed orthopnea.

Physical examination revealed an alert, cyanotic, slightly orthopneic man who weighed 290 pounds and was 67 inches in height. A few rales were heard at the right posterior lung base. The heart was in gallop rhythm, and the pulmonic second sound was accentuated. Mild edema of the abdominal wall and marked edema of the lower extremities was present.

The significant laboratory findings were as follows: red blood cells 6.77 million per cu. mm.; hemoglobin 20 gm. per cent; hematocrit 69 per cent; white blood cells 6,000 per cu. mm. with a normal differential count and normal cellular morphology; platelets 150,000 per cu. mm. The bone marrow showed hyperplasia of the erythroid series, and reticulocytes were 1 per cent of the red blood cells. The blood uric acid was 9.6 mg. per cent. The urinary 17-ketosteroid excretion was 8 mg. in twenty-four hours (normal). X-ray of the chest showed cardiac enlargement and enlargement of the pulmonary vessels. Angiocardiography showed filling of the cardiac chambers in normal sequence. The sella turcica appeared normal by X-ray. An electrocardiogram showed evidence of right ventricular hypertrophy.

Treatment consisted of bed rest, digitalis, sodium and caloric restriction, mercurials and, beginning on the sixth day, repeated phlebotomies. By the end of seventeen days after his date of entry into the hospital a total of five liters of blood had been removed. The patient lost twenty-four pounds in the first five days and an additional thirty pounds before leaving the hospital. He became free of dyspnea and edema and less cyanotic. A chest X-ray taken ten days after admission showed marked diminution in the degree of vascular engorgement, and right ventricular enlargement became apparent. He was discharged on February 9, 1954, to continue on the treatment regimen described above.

His status was re-evaluated on April 4, 1954, when his weight and physical findings were the same as they had been at the time of discharge from the hospital. Red blood cell, white blood cell and platelet counts were normal, and the chest X-ray showed further diminution of vascular markings. Neurological, psychometric and electroencephalographic examinations failed to reveal organic brain disease. However, a slight cerebral dysrhythmia without a focal pattern was present on the electroencephalogram. Intermittent treatment in a chest respirator for several days and advice to breathe deeply did not change his clinical status, and he left the hospital after two weeks of observation.

He entered the hospital again on October 4, 1954, at which time there was no change in the clinical or laboratory findings except for a ten-pound weight gain. During his hospital stay of three weeks, a twenty-pound weight loss was effected by more rigid caloric and sodium restriction.

METHODS

Measurements of lung volumes, maximum ventilatory capacity, ventilation and gas exchange at rest were made according to the methods of Baldwin, Cournand, and Richards (6). Arterial blood was sampled from an indwelling needle in the brachial artery. Oxygen content and capacity and carbon dioxide content were determined by the method of Van Slyke and Neill (7). Whole blood pH was determined at 37° on a Cambridge Research Model pH meter. Expired air was analyzed on the Scholander gas analyzer (8). The oxygen tension of arterial blood was determined by the technique of Riley, Proemmel, and Franke (9). The carbon dioxide tension of arterial blood was obtained by use of the Van Slyke and Sendroy line charts (10). Analysis of the ventilation-perfusion relationships and the diffusion characteristics of the lung at rest was made according to the methods of Riley, Cournand, and Donald (11). The maximal diffusing capacity of the lung was measured by the techniques described by Riley, Shepard, Cohn, Carroll, and Armstrong (12). Cardiac catheterization, sampling of mixed venous blood and determination of cardiac output was carried out as described by Cournand, Baldwin, and Himmelstein (13). During the second cardiac catheterization the procedure for studying a suspected case of congenital heart disease was done according to the method described by the latter authors. Blood pressure in the pulmonary and brachial arteries and in the right ventricle was recorded by the use of calibrated electromanometers. Plasma volume was determined by the T-1824 dye method as described by Gibson, Evans, and Evelyn (14, 15).

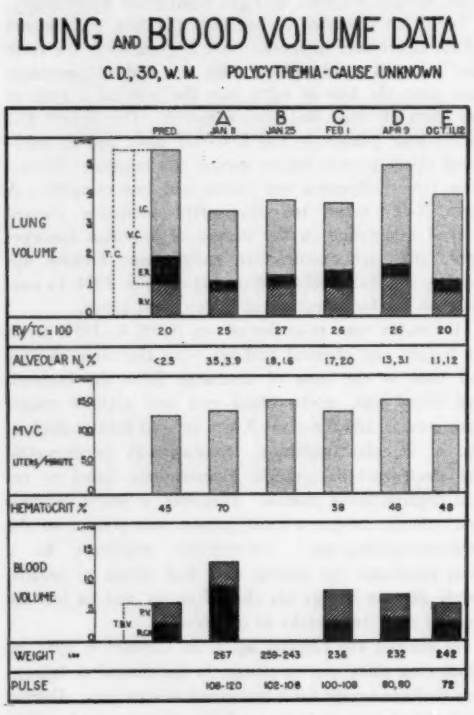

FIGURE 1

Periods: A, before venesection; B, while venesection in progress; C, D, and E, after venesection completed.

The symbols and units used in presenting the observed, assumed and derived values are those recommended by a group of pulmonary physiologists (16) and specifically modified by Riley and Cournand (17).

SYMBOLS

Values for lung volumes, maximum ventilatory capacity and minute ventilation are expressed as liters or liters per minute B.T.P.S. Other symbols and units are as follows:

Ca_{O_2}	O_2 concentration of arterial blood, in vols. per cent.
Cap_{O_2}	O_2 capacity of arterial blood, in vols. per cent.
Ca_{CO_2}	CO_2 content of arterial blood, in vols. per cent.
D_{O_2}	Diffusing capacity of the lungs.
F_{IO_2}	Fractional oxygen concentration of inspired gas.
f	Respiratory rate in breaths per minute.
$P^eA_{O_2}$	"Effective" alveolar P_{O_2} in mm. Hg.
Pa_{O_2}	Arterial P_{O_2} in mm. Hg.
$P^eA_{CO_2}$	"Effective" alveolar P_{CO_2} in mm. Hg. Its equality with Pa_{CO_2} is assumed except where $\dot{Q}va/\dot{Q}t$ exceeds 20 per cent, in which case it is calculated from the venous admixture equation and the physiological CO_2 dissociation curve of the patient.
$\bar{P}a_{CO_2}$	Arterial P_{CO_2} in mm. Hg.
$\bar{P}c_{O_2}$	Mean partial pressure of oxygen in the pulmonary capillaries.
P_{IO_2}	Inspired gas P_{O_2}, in mm. Hg B.T.P.S.
$\dot{Q}va/\dot{Q}t \times 100$	Ratio of venous admixture to total blood flow, expressed as per cent.
R	The CO_2-O_2 exchange ratio or respiratory quotient.
Sa	The oxygen saturation of arterial blood.
Sc^e	The oxygen saturation of "effective" capillary blood.
$S\bar{v}$	The oxygen saturation of mixed venous blood.
$V_D{}^e$	Physiological dead space, corrected for apparatus dead space in ml. B.T.P.S.
$V_T{}^e$	Tidal volume, corrected for apparatus dead space in ml. B.T.P.S.
$V_D{}^e/V_T{}^e$	Dead space-tidal volume fractional ratio.
$\dot{V}_E$	Minute volume of expired gas in liters per minute B.T.P.S.
$\dot{V}_{O_2}$	O_2 consumption in ml. per minute S.T.P.D. (standard temperature and pressure, dry).

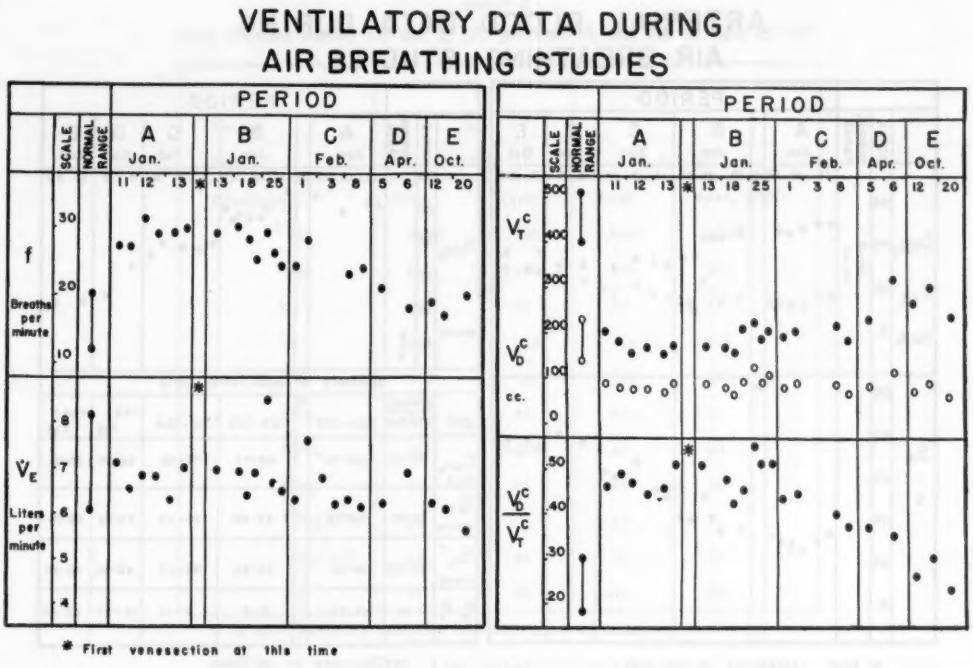

FIGURE 2

Refer to text for definition of symbols.

RESULTS

The results of physiologic study are indicated in Figures 1 to 3 and Tables I to III. Shortly after admission to the hospital, during a period (period A) when rapid weight reduction was occurring, there was a considerable reduction in the total lung capacity which resulted chiefly from a reduction of the vital capacity. Despite the somewhat elevated alveolar nitrogen concentration after the patient had breathed 99 per cent oxygen for seven minutes, it was evident from the reduced absolute value for residual lung volume, the normal maximum ventilatory capacity and the normal spirogram that obstructive emphysema was not present. The estimated total blood volume was almost two times greater than the predicted value and this increase was entirely due to erythrocytosis. Although total minute ventilation was normal, the tidal volume was much reduced. The arterial oxygen tension and oxyhemoglobin per cent saturation were extremely low and carbon dioxide tension was greatly increased. Following the inhalation of 99 per cent oxygen (Table I), a further reduction in tidal volume occurred with a consequent rise in arterial blood carbon dioxide tension, while arterial oxygen saturation increased to 89 per cent. Further study in this initial period (A) revealed that the physiological dead space and the diffusing capacity of the lung at rest were normal (18, 19) but that the percentage of venous admixture was greatly increased. The percentage venous admixture in this instance was calculated according to the equation of Riley, Cournand, and Donald (11) employing the data obtained from cardiac catheterization and the inhalation of 99 per cent oxygen. Studies of the hemodynamics of the right heart and pulmonary circulation revealed elevation of the right ventricular end diastolic pressure and a marked degree of hypertension in the pulmonary artery which did not change immediately after the removal of 600 ml. of blood.

During the next seventeen days (period B), a total of 5 liters of blood was removed so that the

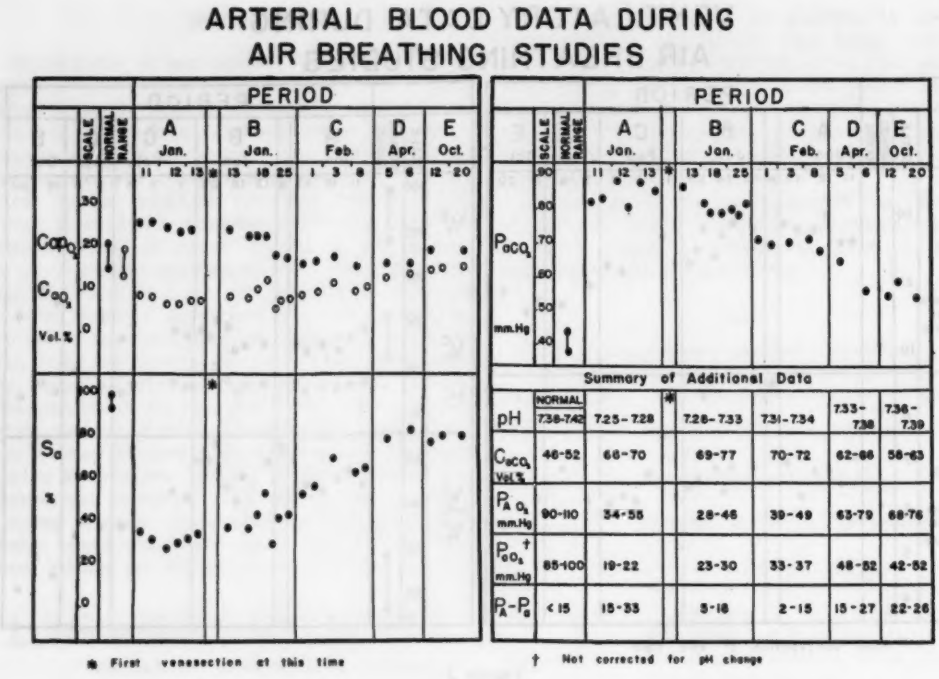

FIGURE 3

Refer to text for definition of symbols.

total blood volume and red cell mass returned to normal. Throughout the ten months of observation sodium and caloric restriction were maintained, and digitalis administration was continued. Repeated study at intervals (periods C, D, and E) showed no significant changes in the measurements of lung volumes and maximum ventilatory capacity, and erythrocytosis did not recur. With the progressive increase in tidal volume, the values for arterial oxygen saturation and tension and carbon dioxide tension returned toward normal, but even after ten months the tidal volume was reduced, and a moderate degree of hypercapnia and arterial hypoxia was present. It is evident from Table I that alveolar ventilation could be much improved by the use of inspiratory positive pressure breathing. The percentage of venous admixture after recovery from failure came to be within normal limits during exercise (20) but was considerably elevated in the last period (E) of study. The maximal diffusing capacity was measured at three and ten months after the initial studies and was found to be normal (20). A second cardiac catheterization (Table III) was done two months after the first one. Although the pressure in the pulmonary artery was lower than before, it was still considerably elevated. In addition, the end diastolic pressure in the right ventricle was still above normal. When 99 per cent oxygen was inhaled during the course of this catheterization, full saturation of the arterial blood with oxygen occurred, but the pressure in the pulmonary artery fell only slightly. Neither an abnormal intracardiac communication nor a valvular stenosis could be demonstrated at this second catheterization.

DISCUSSION

The observations reported here do not permit a classification of this patient's illness into any previously described clinical or physiologic syndrome. The physiologic findings differ from the patterns of dysfunction which have been found

TABLE I

Data obtained during the use of 100 per cent O_2 and the Bennett IPPB *

Period	A		C	
Date	1-12-54		2-8-54	
Condition	Rest	Rest	Rest	Rest, IPPB
$F_{I_{O_2}}$	.2086	.9939	.2086	.2086
Pulse	108	108	104	80
f	29	32	24	31
$\dot{V}_E$	6.8	5.3	6.1	9.2
V_T^c	169	101	185	232
P_{aCO_2}	80	91	67	52
$P^cA_{O_2}$	39	>400	45	84
P_{aO_2}	19	65	33	58
$P^cA_{O_2}-P_{aO_2}$	20		12	26
V_D^c/V_T^c	.44	.46	.37	.38
Sa	30	89	65	87

* In both instances sampling was done after 15 minutes. Values for $P^cA_{O_2}$ and P_{aO_2} are corrected to pH 7.40.

TABLE II

Two level oxygen studies

Period	Status	Date	$P_{I_{O_2}}$	$\dot{V}_E$	$\dot{V}_{O_2}$	R	f	P_{aCO_2}	$P_{aCO_2}-P^cA_{CO_2}$	V_D^c/V_T^c	$\frac{\dot{Q}va}{\dot{Q}t}$ x 100	D_{O_2}	$P^cA_{O_2}$	$P^c_{CO_2}$	P_{aO_2}	$P^cA_{O_2}-P_{aO_2}$	$P^cA_{O_2}-P_{\bar{v}O_2}$	$Sc^e-S\bar{v}$
A	Rest	1-13-54	148	6.3	327	.73	29	88	?	.46	44-67*	>21	32	(17-22)	17	15	<15	(15-25)
C	Rest	2-8-54	237	5.3	351	.59	20	73	(0)	.40	20		109	109	73	36	14	(27)
			145	6.1	336	.64	24	67	(0)	.37	20	24	45	35	33	12	14	(27)
D	Rest	4-5-54	174	6.1	353	.64	21	68	5	.35	33		80	80	51	29	17	(30)
			148	6.2	317	.72	21	65	3	.37	33	19	63	59	44	19	17	(30)
D	Exercise	4-7-54	173	22.5	1488	.78	29	67	(0)	.25	8		86	84	68	18	27	(40)
	3.1 mph at 6°		120	29.7	1531	.92	27	59	(0)	.24	8	57	53	33	32	21	27	(40)
D	Exercise	4-12-54	173	20.2	1616	.75	23	76	(0)	.24	4		70	57	54	16	31	(40)
	3.1 mph at 6°		121	30.5	1543	.96	23	57	(0)	.22	4	50	58	32	32	26	31	(40)
E	Rest	10-12-54	171	6.3	330	.73	17	60	2	.33	32		94	89	54	40	32	(30)
			145	6.1	329	.72	17	59	2	.30	32	10	69	51	40	29	32	(30)
E	Rest	10-21-54	148	5.6	289	.71	19	56	1	.23	32		73	72	49	24	16	(30)
			121	6.4	302	.85	19	49	2	.14	32	19	64	61	45	19	16	(30)
E	Exercise	10-15-54	171	25.4	1767	.76	29	65	(0)	.22	18		86	81	56	30	30	(40)
	3.1 mph at 6°		119	32.3	1699	.93	27	57	(0)	.20	18	57	56	32	29	27	30	(40)

* Calculated from response to 100% O_2 inhalation on preceding day (See Text).
Assumed values are placed in parentheses

TABLE III

Catheterization data before and after therapy

Date, Period	NORMAL AT REST	1ST CATHETERIZATION Jan. 13 (Period A)			2ND CATHETERIZATION Feb. 3 (Period C)			
Condition		Rest (Control)		After 600 cc Venesection	Rest (Control)			After 100% O_2 for 15 Minutes
BSA (m^2)		2.27			2.17			
Weight (lbs.)		266			236			
Height (cm.)		170			170			
Heart Rate (beats/min.)		108	120	106	94			84
Stroke Volume (cc/beat)		85	93	108	125			
Cardiac Output (liters/min.)	7.0	9.1	11.1	11.4	11.5			
A.V. Difference (vols.%)	4-5	3.6	3.0	2.9	3.1			
O_2 Consumption (cc/min.)		327	336	332	359			
R.Q.		.73	.74	.74	.72			
Pressures (mm. Hg):								
Pulm. Art. S/D	30/10	104/50	96/50	94/55	92/49	79/44	59/35	56/33
Mean	15	67	65	66	64	60	47	41
Rt. Vent. S/D	30/5			108/21			66/15	-
Rt. Aur. Mean	5			22			15	-
Brach. Art. S/D		144/92	136/84	115/64	124/78	132/66	141/65	146/80
$P\bar{a}co_2$ (mm. Hg)	40	88	86	87	73			76
Sa (%)	94-98	32	32	37	68			100
$S\bar{v}$ (%)		17	19	24	51			93

to characterize those groups of pulmonary diseases that have been described under the heading of pulmonary fibrosis (21), alveolar-capillary block (22), or pulmonary emphysema (23). The observations are similar in most respects to those made by Newman, Feltman, and Devlin (5) in two individuals, and it seems likely that the pathogenesis of the illness is the same in the two instances. However, the failure of the maximum ventilatory capacity to return to normal after treatment in one of their cases contrasts to the ever normal value in this individual and suggests that their patient may have had an additional ventilatory defect.

The severe degree of alveolar hypoventilation demonstrated by this patient can probably account for all of the other physiologic abnormalities which were observed. Thus, arterial hypoxia and carbon dioxide retention would be expected as the immediate consequence of such a defect. With continued hypercapnia and the secondary rise in buffer base the respiratory center would become less sensitive to carbon dioxide, and hence the hypoventilation would tend to be perpetuated. It seems likely that the very small tidal volume which characterized this hypoventilation would leave areas of the lung whose blood circulation was maintained without adequate ventilation and in this manner account for the elevated percentage of venous admixture that was found. It would not be unexpected that the persistence of such hypoxia over a long period would lead to polycythemia. Finally, from the report of similar findings in a patient with diffuse disease of the central nervous system but without intrinsic pulmonary disease (24), it seems possible that long standing hypoxia with its consequent polycythemia may have been sufficient cause for pulmonary hypertension and right heart failure of the high output type to occur.

It is evident from the data presented that no abnormal right to left circulatory shunt existed. Although the percentage of venous admixture was extremely high initially and full saturation of the arterial blood on 99 per cent oxygen breathing was not achieved, it seems likely that this resulted from the marked pulmonary congestion. Neither exploratory cardiac catheterization nor angiocardiography revealed a shunt, and in subsequent study the percentage venous admixture was much reduced and during exercise approached normal values. In addition, increased venous admixture

would offer no explanation for the CO_2 retention observed (11, 25).

Whether or not the sequence of events was that which has been postulated above, an initiating mechanism for the alveolar hypoventilation was not discovered. It has been suggested (5) that polycythemia vera by a direct effect on the respiratory center might cause hypoventilation, but in this case no confirmatory manifestations of polycythemia vera, *e.g.*, splenomegaly, leucocytosis or thrombocytosis were present. It also seems unlikely that primary damage to the respiratory center with consequent loss of carbon dioxide sensitivity was present since no other signs of organic brain disease could be found and since arterial carbon dioxide tension returned toward normal following treatment. Since the hypoventilation was characterized by a marked reduction of tidal volume, it is possible that it resulted from an overabundance of inspiratory-inhibitory impulses to the respiratory center from stretch receptors either in the lungs or elsewhere in the ventilatory apparatus. If disease of the pulmonary parenchyma accounted for such a disturbance, it was not of sufficient extent to result in detectable roentgenological abnormality. In the absence of such abnormality, the reduction in total lung capacity can best be related to extreme obesity, cardiac enlargement and a long standing pulmonary vascular disturbance. The patient's exposure to fumes from aluminum furnaces was of only six months' duration; this fact, together with the lack of radiologic abnormality would seem to rule out the presence of a pneumoconiosis of the type described by Shaver and Riddell (26).

It appears possible, however, that the obesity either by a mechanical effect leading to an increase in the work of breathing or else by a reflex effect might give rise to a diminished tidal volume. With respect to mechanical effects it is known that the addition of resistance to the airway (27) can induce progressively decreasing tidal volume with carbon dioxide retention. This patient had no increase in airway resistance as evidenced by the normal maximum ventilatory capacity and spirograms. Undoubtedly there are other factors, both mechanical and reflex, such as the retractive forces and stretch receptors of the chest bellows which could modify tidal volume, but satisfactory methods of studying these in patients have not been developed. Therefore, the importance of the obesity in this instance cannot be evaluated.

Severe pulmonary hypertension at rest was present even after all clinical evidence of right heart failure had disappeared. The elevation of cardiac output was not of sufficient magnitude to cause pulmonary hypertension in a normal individual (28). Since correction of the arterial oxygen unsaturation did not relieve the pulmonary hypertension, it was probably not maintained by hypoxia alone. Thus it seems probable that the pulmonary hypertension was at least in part due to an anatomic reduction in the capacity of the pulmonary vascular bed. It is likely that such anatomic reduction was in the arteriolar or arterial branches since the normal maximum diffusing capacity suggests that the area of the pulmonary capillary surface was normal (12). It is impossible to determine with certainty whether pulmonary arterial disease resulted from long standing hypoxia or was primary. However, if primary pulmonary vascular disease was present, it was not of the type described by Dresdale, Schultz, and Michtom (29), for their patients, unlike this one, had a reduced cardiac output and normal arterial oxygen saturation.

SUMMARY

An obese young man with cyanosis, dyspnea and edema was found to have erythrocytosis and right ventricular hypertrophy but no evidence of disease of the lungs or chest wall or of the central nervous system. Physiologic study revealed the presence of marked arterial hypoxia and hypercapnia and severe alveolar hypoventilation which was characterized by a marked reduction in tidal volume without an increase in respiratory frequency sufficient to maintain normal alveolar ventilation. Marked pulmonary arterial hypertension and increased cardiac output were also present. There was no evidence that these findings were initiated by an abnormal cardiac or vascular communication. Treatment with bed rest, low salt diet, mercurials, digitalis, and phlebotomy was associated with disappearance of dyspnea and edema. When studied after ten months of such treatment arterial hypoxia and hypercapnia and alveolar hypoventilation were still present al-

though to a lesser degree than at the time of initial observation.

All of the physiologic abnormalities have been attributed to the alveolar hypoventilation, but an initiating mechanism for the alveolar hypoventilation was not discovered.

Addendum

Since this paper was submitted for publication two additional reports have appeared in which very similar physiologic derangements are described (30, 31).

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THE EFFECTS OF EXERCISE ON CARDIOVASCULAR AND RENAL FUNCTION IN CARDIAC PATIENTS WITH AND WITHOUT HEART FAILURE[1]

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Exercise in the upright position results in the retention of salt and water in normal subjects (1–5) and in patients with congestive failure (6). The mechanisms which set off these antidiuretic and antisaluretic responses are not definitely known. The following factors have been considered: 1) Sensory stimuli from the legs; 2) upright position; 3) hemoconcentration; 4) reduction and/or redistribution of the effective circulating blood volume; 5) metabolic products or alteration of acid-base balance; and 6) a relatively inadequate cardiac output resulting in a tendency to hypotension or collapse. Studies in this laboratory on normal subjects in the supine position showed that strenuous exercise (alternate straight leg-raising) does not regularly produce such antidiuretic and antisaluretic responses (7). In the present investigation the study was extended to include patients with valvular and non-valvular forms of heart disease with or without congestive failure, and patients with chronic pulmonary disease with or without congestive failure, to determine: 1) Whether exercise in the supine position in these patients causes antidiuretic and antisaluretic responses; and 2) whether there is a consistent relationship between the cardiodynamic and the renal responses to such exercise.

PATIENTS AND METHODS

Group A consisted of fifteen patients with severe valvular and non-valvular heart disease. All patients in this group were considered to have some degree of circulatory failure as indicated by an impaired cardiac output response during exercise, by an abnormal right ventricular end diastolic pressure of over 8 mm. Hg, or both. Nine of the twelve patients with rheumatic heart disease (mitral stenosis and/or mitral insufficiency and minimal aortic disease) had clinical and cardiodynamic evidence of right ventricular failure. Two patients with arteriosclerotic heart disease and one patient with congenital heart disease and associated pulmonary vascular disease had congestive failure. Five of the patients (A. L., C. S., C. J., F. A., J. C.) had minimal to moderate peripheral edema. All patients with right ventricular failure and/or auricular fibrillation were on maintenance oral digitalis therapy and in some cases were receiving mercurial diuretics.

Group B consisted of eight patients with chronic pulmonary disease. Three of these patients (W. D., M. A., W. H.) had classical evidence of right ventricular hypertrophy by electrocardiogram and roentgenogram of the heart as well as hemodynamic evidence of right heart failure. In three others (M. M., D. K., C. Di.) electrocardiographic or roentgenographic evidence (or both) of right ventricular enlargement was lacking, but the hemodynamic measurements of right ventricular filling pressure indicated mild right ventricular failure, particularly during exercise. The remaining two patients (J. M., M. G.) had pulmonary arterial pressures which were elevated at rest or rose during exercise, but no evidence of congestive failure.

The patients were studied in the post-absorptive state without sedative medication. The experimental procedure and methods of measurement of cardiovascular and renal functions were similar to those previously described (8).

RESULTS

Individual cardiovascular and renal data at rest and in response to exercise are presented in Tables I and II; detailed data on seven other patients (A. L., C. S., E. M., C. M., E. T., A. C., J. C.) with predominately mitral stenosis have already been published (8). The means for all (including these seven) patients together with a statistical analysis are shown in Table III.

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TABLE I

Individual cardiodynamic data

Patient Age Sex Body surface area (m^2)	Diagnosis and clinical classification*	Procedure	Cardiac index *L./min./m^2*	Oxygen consumption *ml./min./m^2*	A-V oxygen difference *Vol. %*	Arterial oxygen saturation %	Hematocrit (arterial) %	Pulmonary artery† S	Pulmonary artery† D	Pulmonary artery† M	Pressures *mm. Hg* "Pulmonary capillary" M	Pressures *mm. Hg* Right ventricular end diastolic	Systemic arterial S	Systemic arterial D	Systemic arterial M	Pulse rate *beats/min.*	Resistances *dyncs seconds cm^{-5}* "Total pulmonary"	Resistances *dyncs seconds cm^{-5}* "Pulmonary arteriolar"	Resistances *dyncs seconds cm^{-5}* Total peripheral
Group A—Valvular and Non-valvular Heart Disease																			
I. C. 42 F 1.65	R.H.D.-M.S. NSR III	Control	2.72	130	4.79	94	39	28	14	18	14	4	105	75	85	82	320	70	1,510
		Exercise 7 min.‡	3.93	261	6.63	93	39	41	15	22	20	8	116	80	92	108	270	20	1,130
J. Di. 38 M 1.69	R.H.D. MI A.F. IV	Control	1.60	139	8.70	95	40	50	32	36	—	14	110	80	90	88	1,070	—	2,660
		Exercise 5 min.	3.21	389	12.12	98	41	80	38	50	—	18	125	80	100	136	740	—	1,470
V. C. 32 F 1.54	R.H.D. MI A.F. III	Control	2.19	145	6.59	95	38	40	—	—	—	12	110	65	85	82	—	—	2,010
		Exercise 6 min.	3.65	371	10.20	94	38	65	—	—	—	30	140	80	100	160	—	—	1,420
C. J. 49 M 1.93	R.H.D.-M.S. A.S.H.D. A.F. III	Control	1.99	143	7.16	96	48	50	20	30	—	12	160	90	105	87	620	—	2,180
		Exercise 5 min.	3.09	358	11.60	100	48	60	25	38	—	15	160	90	105	126	510	—	1,410
A. F. 32 F 1.53	R.H.D. MI A.F. IV	Control	1.46	139	9.51	96	48	43	25	30	26	7	110	62	80	90	1,070	140	2,850
		Exercise 7 min.	1.82	222	12.18	100	48	62	38	48	35	10	135	75	90	129	1,370	370	2,580
A. J. 53 F 1.45	A.S.H.D. A.F. III	Control	1.95	120	6.13	97	44	29	17	23	8	3	145	70	95	74	640	420	2,660
		Exercise 5 min.	2.50	268	10.72	100	44	68	30	50	17	5	176	80	120	115	1,100	730	2,640
F. A. 61 F 1.85	A.S.H.D. A.F. IV	Control	2.10	122	5.78	97	54	60	40	45	—	18	118	66	84	92	920	—	1,720
		Exercise 9 min.	2.46	266	10.79	98	56	85	57	62	—	22	128	78	90	116	1,090	—	1580
M. D. 50 F 1.58	C.H.D. A.S.D. N.S.R. III	Control	2.56	137	5.35	94	44	78	31	55	15	11	130	75	100	86	1,090	790	1,980
		Exercise 5 min.	—	326	—	82	45	140	48	85	30	15	200	110	150	118	—	—	—

Additional data on seven patients (A. L., C. S., E. M., C. M., E. T., A. C., J. C.) have been presented in another publication (13) to which the reader is referred.

* R.H.D. = Rheumatic heart disease; A.S.H.D. = Arteriosclerotic heart disease; H.C.V.D. = Hypertensive cardiovascular disease; C.H.D. = Congenital heart disease; A.S.D. = Atrial septal defect; P.F. = Pulmonary fibrosis; P.E. = Pulmonary emphysema; C.P. = Cor pulmonale; P.H.A. = Posthemorrhagic anemia; A.V.F. = Arteriovenous fistula; A.F. = Auricular fibrillation; N.S.R. = Normal sinus rhythm; M.S. = Mitral stenosis; M.I. = Mitral insufficiency; m^2—refers to square meters of body surface area.

† S = Syatolic; D = Diastolic; M = Mean.

‡ Duration of exercise.

TABLE I—*Continued*

Patient Age Sex Body surface area (m^2)	Diagnosis and clinical classification*	Procedure	Cardiac index *L./min./m^2*	Oxygen consumption *ml./min./m^2*	A-V oxygen difference *Vol. %*	Arterial oxygen saturation %	Hematocrit (arterial) %	Pulmonary artery† S	D	M	Pressures *mm. Hg* "Pulmonary capillary" M	Right ventricular end diastolic	Systemic arterial S	D	M	Pulse rate *beats/min.*	Resistances *dynes seconds cm^{-5}* "Total pulmonary"	"Pulmonary arteriolar"	Total peripheral
Group B—Chronic Pulmonary Disease																			
J. M. 63 M 1.59	P.F. P.E. N.S.R. II	Control	3.15	162	5.15	95	42	26	12	16	10	2	155	65	100	74	260	100	1,600
		Exercise 5 min.	6.38	514	8.05	94	43	30	14	20	10	4	220	80	140	86	160	80	1,100
M. M. 22 M 1.75	P.F. C.P. N.S.R. II	Control	3.05	154	5.06	87	40	53	30	38	—	8	120	90	102	92	570	—	1,530
		Exercise 6 min.	5.33	368	6.90	87	41	70	40	48	—	12	148	100	120	116	410	—	1,030
D. K. 49 M 1.86	P.E. C.P. N.S.R. II	Control	3.78	171	4.55	89	45	40	24	28	7	5	160	100	125	82	320	240	1,420
		Exercise 6 min.	6.05	434	7.18	87	46	60	35	42	10	10	180	100	130	105	300	230	920
C. Di. 63 M 1.85	P.E. C.P. N.S.R. II	Control	3.39	140	4.13	90	57	50	27	35	13	8	140	65	90	60	450	280	1,150
		Exercise 8 min.	6.02	467	7.76	94	58	60	30	39	—	20	150	70	96	82	280	—	690
M. G. 67 F 1.66	P.E. C.P. N.S.R. II	Control	2.80	131	4.68	91	48	33	18	25	12	5	135	70	100	84	430	220	1,720
		Exercise 5 min.	4.31	368	8.55	90	48	55	35	40	—	—	160	80	115	100	450	—	1,290
W. D. 66 M 1.76	P.F. C.P.§ N.S.R. III	Control	3.65	137	3.75	52	50	81	42	53	18	23	150	75	100	92	660	440	1,250
		Exercise 5 min.	3.50	215	6.12	51	51	110	58	68	18	30	210	90	130	112	880	650	1,680
M. A. 46 M 1.55	P.F. C.P.§ N.S.R. IV	Control	2.84	116	4.09	46	50	98	55	70	20	18	145	70	90	98	1,270	910	1,640
		Exercise 3 min.	4.47	195	4.38	42	51	108	58	76	22	32	170	85	110	98	880	620	1,270
W. H. 56 M 2.00	P.E. C.P.§ N.S.R. IV	Control	3.49	154	4.42	75	71	60	44	50	18	22	130	80	90	94	570	370	1,030
		Exercise 4 min.	3.84	283	7.37	67	72	75	50	60	25	25	120	60	85	110	620	360	880
Group C—"High Cardiac Output" Disease																			
M. S. 44 M 1.77	H.C.V.D. P.H.A. N.S.R. I	Control	4.69	147	3.14	99	20	36	21	26	10	10	220	110	140	88	250	150	1,350
		Exercise 5 min.	7.10	369	5.21	100	22	60	30	43	25	20	280	120	160	124	270	110	1,020
H. H. 31 F 1.61	A.V.F. N.S.R. III	Control	5.04	152	2.76	92	30	43	23	31	20	13	100	50	70	88	310	180	690
		Exercise 7½ min.	8.51	329	3.87	93	31	63	28	40	26	—	120	60	90	112	230	—	520

§ During congestive failure.

Cardiovascular responses

Cardiac index and oxygen measurements. At rest the mean cardiac index for the patients with valvular and non-valvular heart disease (Group A) was 2.46 L. per min. per m². Eleven of the fifteen patients in this group had abnormally low cardiac indices (< 2.75 L. per min. per m²). In contrast, all of the patients with chronic pulmonary disease with or without congestive failure (Group B) had normal cardiac indices at rest, the average being 3.27 L. per min. per m². The mean difference of 0.81 L. per min. per m² between the resting cardiac indices of these two groups was highly significant statistically. However, in response to exercise the mean increase in cardiac index for both groups was of similar magnitude and represented a highly significant change (average 1.08 L. per min. per m² or 44 per cent for Group A and 1.72 L. per min. per m² or 53 per cent for Group B). Although the response of the cardiac index to exercise in Group B was 0.63 L. per min. per m² greater, this difference was not statistically significant.

Oxygen consumption at rest and in response to exercise was similar in the two groups. This suggested that the two groups performed comparable amounts of work during exercise. The mean arteriovenous oxygen difference in Group A was abnormally elevated at rest (mean 6.01 vol. per cent) and during exercise (10.14 vol. per cent). On the other hand, the mean arteriovenous oxygen difference in Group B was within the normal

TABLE II

Individual renal data

Patient	Diagnosis and clinical classification	Procedure	Periods	C_{PAH} *ml./min./1.73 m²*	C_{IN} *ml./min./1.73 m²*	F.F. %	UV *ml./min.*	$U_{Na}V$ *microEq./min.*	$U_{Cl}V$ *microEq./min.*	$U_{K}V$ *microEq./min.*
Group A—Valvular and Non-valvular Heart Disease										
I. C.	R.H.D.	Control	1–4	350	93	27	5.8	24	24	24
	M.S.	Exercise	5	215	58	27	1.3	11	17	19
	N.S.R. III	7 min. Recovery	6–7	248	75	30	0.8	12	16	27
J. D.	R.H.D.	Control	1–3	206	94	45	2.1	132	144	109
	M.I.	Exercise	4	106	44	41	1.7	66	78	53
	A.F. IV	5 min. Recovery	5–6	208	101	49	0.6	30	63	79
V. C.	R.H.D.	Control	1–4	477			6.2	22	42	53
	M.I.	Exercise	5	207			6.7	18	30	25
	A.F. III	6 min. Recovery	6–8	343			3.3	16	33	32
C. J.*	R.H.D.	Control	1–3	279	104	37	0.7	32	39	36
	M.S.	Exercise	4	332	123	40	0.7	20	40	45
	A.S.H.D. A.F. III	5 min. Recovery	5–6	246	106	43	0.9	24	36	52
A. F.	R.H.D.	Control	1–4	357			6.9	9	72	21
	M.I.	Exercise	5	268			5.9	7	56	21
	A.F. IV	7 min. Recovery	6–8	289			5.8	8	53	21
A. J.	A.S.H.D.	Control	1–5	287	106	37	8.4	196	130	55
	A.F.	Exercise	6	268	100	37	0.6	63	44	50
	III	5 min. Recovery	7–8	295	98	33	0.5	49	42	60
F. A.	A.S.H.D.	Control	1–3	143	56	39	0.8	35	58	43
	A.F.	Exercise	4	130	46	36	0.4	22	44	40
	IV	9 min. Recovery	5–6	151	60	40	0.5	30	53	51
M. D.	C.H.D.	Control	1–4	193	89	46	4.9	69	64	41
	I.A.S.D.	Exercise	5	143	65	45	0.9	30	42	35
	N.S.R. III	5 min. Recovery	6	194	81	42	0.4	22	19	32

TABLE II—*Continued*

Patient	Diagnosis and clinical classification	Procedure	Periods	C_{PAH} *ml./min./1.73 m²*	C_{IN} *ml./min./1.73 m²*	F.F. %	UV *ml./min.*	$U_{Na}V$ *microEq./min.*	$U_{Cl}V$ *microEq./min.*	$U_{K}V$ *microEq./min.*
			Group B—Chronic Pulmonary Disease							
J. M.	P.F.	Control	1–4	292	85	30	1.8	84	128	46
	P.E.	Exercise	5	183	66	36	0.3	29	60	31
	N.S.R.	5 min.								
	II	Recovery	6–7	272	91	33	0.8	57	96	52
M. M.	P.F.	Control	1–3	266	84	31	2.6	192		90
	C.P.	Exercise	4	180	77	43	1.6	166		70
	N.S.R.	6 min.								
	II	Recovery	5–6	208	91	43	1.9	186		152
D. K.	P.E.	Control	1–4	438	112	26	1.8	148	152	86
	C.P.	Exercise	5	433	126	29	1.5	128	148	82
	N.S.R.	6 min.								
	II	Recovery	6–7	419	115	27	1.4	128	132	81
C. D.	P.E.	Control	1–3	347	92	26	0.6	23	11	23
	C.P.	Exercise	4	319	100	31	0.8	20	14	24
	N.S.R.	8 min.								
	II	Recovery	5–7	372	104	27	3.0	17	18	41
M. G.	P.E.	Control	1–3	176	51	30	2.1	95	64	48
	C.P.	Exercise	4	173	51	30	2.0	67	52	25
	N.S.R.	5 min.								
	II	Recovery	5–7	170	51	30	1.9	45	35	38
W. D.	P.F.	Control	1–3	212	91	43	1.2	22	48	53
	C.P.*	Exercise	4	106	58	54	0.6	27	40	28
	N.S.R.	5 min.								
	III	Recovery	5–7	198	93	50	0.4	20	41	48
M. A.	P.F.	Control	1–3	349			1.0	241	217	97
	C.P.*	Exercise								
	N.S.R.	3 min.								
	IV	Recovery	5–7	332			1.7	161	162	94
W. H.	P.E.	Control	1–3	212	91	43	0.7	28	46	51
	C.P.*	Exercise								
	N.S.R.	4 min.								
	IV	Recovery	5–6	202	89	44	0.3	7	32	45
			Group C—"High-Cardiac-Output" Disease							
M. S.†	H.C.V.D.	Control	1–3	452	100	22	12.0	890		93
	P.H.A.	Exercise	4	403	96	24	7.6	730		78
	N.S.R.	5 min.								
	I	Recovery	5–7	414	96	23	3.7	297		62
H. H.	A.V.F.	Control	1–4	289	93	32	1.4	12	12	79
	N.S.R.	Exercise	5	230	76	33	0.5	6	5	61
	III	7½ min.								
		Recovery	6–8	291	98	34	0.8	13	5	79

* During congestive failure.
† Patient was given 300 ml. 5 per cent NaCl I.V.

range at rest (4.47 vol. per cent) and during exercise (7.04 vol. per cent). The differences between the values of the two groups at rest and during exercise and the change in values in response to exercise were all highly significant statistically. Thus, the increases in cardiac output during exercise in Group A were not commensurate with the associated rises in oxygen consumption so abnormally elevated mixed arteriovenous oxygen differences resulted. In the patients in Group B, however, the increases in cardiac output during exercise were comparable with the associated rises in oxygen consumption so the arteriovenous oxygen differences showed normal increases.

The mean arterial oxygen saturation for Group

TABLE III

Mean data and statistical analysis

	Cardiac index *L./min./m²*				Oxygen consumption *L./min./m²*			
	Rest	Exercise	Response to exercise	P	Rest	Exercise	Response to exercise	P
Group A	2.46±0.14	3.56±0.26	+1.08±0.14	<0.01	141±5	348±20	+206±17	<0.01
Group B	3.27±0.10	4.98±0.40	+1.72±0.40	<0.01	146±6	356±41	+210±37	<0.01
Diff.	+0.81±0.17	+1.42±0.47	+0.63±0.42	—	+5±8	+8±46	+4±41	—
P	<0.01	0.06	0.1	—	0.5	0.9	>0.9	—

	A-V O_2 difference *Vol. %*				Arterial oxygen saturation *%*			
	Rest	Exercise	Response to exercise	P	Rest	Exercise	Response to exercise	P
Group A	6.01±0.39	10.14±0.52	+4.09±0.33	<0.01	95±3	95±2	+0.4±0.9	0.7
Group B	4.47±0.17	7.04±0.46	+2.56±0.40	<0.01	78±7	76±7	−2±1	0.2
Diff.	−1.54±0.42	−3.10±0.69	−1.53±0.51	—	−17±7	−19±8	−2.4±1.5	—
P	<0.01	<0.01	<0.01	—	0.03	0.02	0.2	—

	Hematocrit *%*				Pressures: Mean pulmonary arterial *mm. Hg*			
	Rest	Exercise	Response to exercise	P	Rest	Exercise	Response to exercise	P
Group A	42±2	44±1	+1±0.3	<0.01	38±4	60±6	+22±3	<0.01
Group B	50±4	51±4	+1±0.1	<0.01	39±6	49±6	+10±2	<0.01
Diff.	+8±4	+7±4	0±0.3	—	+1±8	−11±8	−12±4	—
P	0.05	0.09	0.1	—	0.9	0.2	<0.01	—

	Pressures: Mean "pulmonary capillary" *mm. Hg*				Pressures: Right ventricular end diastolic *mm. Hg*			
	Rest	Exercise	Response to exercise	P	Rest	Exercise	Response to exercise	P
Group A	22±2	36±4	14±2	<0.01	9±1	15±2	+6±1	<0.01
Group B	14±2	17±3	+2±1	0.1	11±3	19±4	+7±2	<0.01
Diff.	−8±3	−19±5	−12±3	—	+2±3	+4±5	+1±2	—
P	0.02	<0.01	<0.01	—	0.6	0.4	0.8	—

	Pressures: Mean systemic *mm. Hg*				Pulse rate *beats/min.*			
	Rest	Exercise	Response to exercise	P	Rest	Exercise	Response to exercise	P
Group A	95±3	117± 8	+22±6	<0.01	80±2	125±6	+45±5	<0.01
Group B	100±2	116± 6	+15±5	0.02	84±6	101±5	+17±2	<0.01
Diff.	+5±5	−1±10	−7±8	—	+4±6	−24±7	−28±6	—
P	0.3	>0.9	0.4	—	0.5	<0.01	<0.01	—

	Resistances *dynes seconds cm⁻⁵*: "Total pulmonary"				"Pulmonary arteriolar"			
	Rest	Exercise	Response to exercise	P	Rest	Exercise	Response to exercise	P
Group A	763± 82	813±110	+113±62	0.09	306± 72	348± 94	+112±41	0.03
Group B	565±112	497± 96	− 68±64	0.3	364±100	388±111	− 21±78	0.8
Diff.	−198±140	−316±146	−181±89	—	+58±123	+40±145	−133±88	—
P	0.2	0.05	0.06	—	0.6	0.8	0.2	—

	Resistances *dynes seconds cm⁻⁵*: Total peripheral			
	Rest	Exercise	Response to exercise	P
Group A	1,948±146	1,550±156	−348±102	<0.01
Group B	1,415± 89	1,108±107	−308±114	0.03
Diff.	−533±171	−342±189	+ 40±173	—
P	<0.01	0.03	0.8	—

TABLE III—*Continued*

	Clearance *ml./min./1.73 m²*									
	PAH					Inulin				
	Rest	Exercise	Response to exercise	P	Recovery	Rest	Exercise	Response to exercise	P	Recovery
Group A	315±27	216±21	−99±20	<0.01	275±22	99± 5	71± 8	−28± 7	<0.01	96± 5
Group B	286±32	232±50	−56±20	0.04	272±33	86± 8	80±11	− 6± 7	0.4	90± 8
Diff.	−29±42	+16±54	+43±29	—	−3±94	−13±10	+9±14	+22±10	—	−6±10
P	0.5	0.8	0.1	—	>0.9	0.2	0.5	0.05	—	0.6

	F.F. %					UV *ml./min.*				
	Rest	Exercise	Response to exercise	P	Recovery	Rest	Exercise	Response to exercise	P	Recovery
Group A	34±2	35±3	+1±2	0.6	37±2	5.5±1.1	2.4±0.6	−3.2±0.7	<0.01	2.4±0.7
Group B	33±2	37±4	+6±2	0.03	36±4	1.5±0.2	1.1±0.3	−0.6±0.2	0.1	1.4±0.3
Diff.	−1±3	+2±5	+5±3	—	−1±4	−4.0±1.2	−1.3±0.6	+2.6±0.7	—	−1.0±0.8
P	0.8	0.7	0.08	—	0.8	<0.01	0.05	<0.01	—	0.2

	$U_{Na}V$ *microEq./min.*					U_KV *microEq./min.*				
	Rest	Exercise	Response to exercise	P	Recovery	Rest	Exercise	Response to exercise	P	Recovery
Group A	90±19	54±11	−36±13	0.02	67±22	49± 6	36± 3	−13±5	0.01	47± 5
Group B	104±29	73±25	−21± 9	0.05	78±25	62± 9	43±10	−14±5	0.03	69±14
Diff.	+14±35	+19±27	+15±15	—	+11±11	+13±11	+7±11	− 1±6	—	+22±15
P	0.7	0.5	0.3	—	0.3	0.3	0.5	>0.9	—	0.2

	$U_{Cl}V$ *microEq./min.*				
	Rest	Exercise	Response to exercise	P	Recovery
Group A	86±11	59± 9	−26±10	0.02	67±15
Group B	95±28	63±23	−18±13	0.2	74±21
Diff.	+9±30	+4±24	+ 8±16	—	+7±26
P	0.8	0.9	0.6	—	0.8

A was within the normal range both at rest and in response to exercise although some individual measurements were slightly below normal. All of the patients except one in Group B had abnormally low arterial oxygen saturations, the average being 78 per cent at rest. There was no significant change in response to exercise (average 76 per cent). The difference in the mean arterial oxygen saturations between the two groups was statistically significant both at rest and during exercise. The mean hematocrit in Group A at rest was within the normal range (average 42 per cent); whereas the mean hematocrit in Group B was abnormally elevated at rest (average 50 per cent), a difference which was statistically significant. The slight increases in hematocrit in both groups during exercise were highly significant statistically, but were not significantly different in the two groups.

Vascular pressures

Pulmonary arterial pressure. All of the patients in Group A had abnormally elevated mean pulmonary arterial pressures both at rest (average 38 mm. Hg) and during exercise (average 60 mm. Hg). Similarly in Group B all except one patient (J. M.) had elevated mean pulmonary arterial pressures at rest (average 39 mm. Hg) and during exercise (average 49 mm. Hg). Although the mean pulmonary arterial pressure during exercise was, on the average, 11 mm. Hg lower in the patients in Group B than Group A, the variation in the individual data was so great that this

difference was not statistically significant. In response to exercise, the mean pulmonary arterial pressure rose 22 mm. Hg in Group A and only 10 mm. Hg in Group B; this difference was highly significant both physiologically and statistically.

"Pulmonary capillary" pressure. At rest the mean "pulmonary capillary" (PC) pressure in all but one patient (A. J.) in Group A was above normal (average 22 mm. Hg). In Group B five of the seven patients in whom the mean "PC" pressure was measured had values that were above or near the upper limits of normal (average 14 mm. Hg). The difference between the groups, averaging 8 mm. Hg, was highly significant. In response to exercise the mean "PC" pressure rose on the average by 14 mm. Hg (to 36 mm. Hg) in Group A, but by only 2 mm. Hg (to 17 mm. Hg) in Group B. This difference was also highly significant.

Right ventricular end diastolic pressure

In nine of the fifteen patients in Group A the right ventricular end diastolic (R.V.D.) pressure was elevated at rest (the average for the entire group being 9 mm. Hg); in five of the patients in Group B it was likewise elevated (average 11 mm. Hg). During exercise the average R.V.D. pressure rose by 15 mm. Hg in Group A and 19 mm. Hg in Group B. The average increases in R.V.D. pressure in response to exercise were approximately equal in the two groups, and the difference between them was not statistically significant.

Arterial pressure. The mean arterial pressure at rest was within the normal range for both Group A (average 95 mm. Hg) and Group B (average 100 mm. Hg). In both groups the mean arterial pressure showed a highly significant increase during exercise. However, the response of the arterial pressure to exercise was not significantly different between the two groups.

Heart rate. The heart rate at rest averaged 80 beats per minute in Group A and 84 beats per minute in Group B. However, the increase in heart rate in response to exercise was, on the average, 28 beats per minute greater in Group A than in Group B. This highly significant difference in response to exercise may have resulted from inadequate digitalization and stabilization of the heart rate in several patients in Group A who had auricular fibrillation.

Vascular resistances (in units of dynes seconds cm.$^{-5}$)

Vascular resistances were calculated according to formulae previously discussed and defined (9). The so-called "total pulmonary" resistance, which actually includes the total resistance opposing the right ventricle, was elevated at rest in Group A (average 763 units) and in Group B (average 565 units); the difference was not statistically significant. In response to exercise total pulmonary resistance rose in Group A (113 units) while it decreased on the average 68 units in Group B. This difference proved to be just significant statistically.

The "pulmonary arteriolar" resistance, actually the total pulmonary vascular resistance, was calculated to be increased at rest in both Group A (average 306 units) and Group B (average 364 units). This difference was not statistically significant. However, there was an average increase of 112 units in "pulmonary arteriolar" resistance in response to exercise in nine patients in Group A while there was an average decrease of 21 units in Group B. The difference in the two groups in response to exercise was not significant by statistical analysis.

Since the cardiac output was significantly lower in the Group A patients than in Group B, their mean arterial pressures were maintained at equal levels as a result of a greater *total peripheral resistance* (1,948 units compared with 1,415 units in Group B). This difference was highly significant statistically. However, in response to exercise the decrease in total systemic resistance in the two groups was not significantly different.

Renal responses

At rest. The renal plasma flow was abnormally low in all the patients in both Group A (mean 315 ml. per min. per 1.73 m^2) and Group B (mean 286 ml. per min. per 1.73 m^2). Glomerular filtration rates, measured in thirteen of the Group A patients, averaged 99 mm. per min. per 1.73 m^2, which is abnormally low. Seven of these patients had low glomerular filtration rates, and six were within the range of normals. In contrast, six of the seven patients in Group B in whom this function was measured had abnormally low values, the average for the group being 86

ml. per min. per 1.73 m² (9).[4] Although the average renal plasma flow and glomerular filtration rate measurements tended to be lower in Group B than Group A, the differences were not statistically significant. Inasmuch as the dietary intake of salt was not uniformly controlled in the patients in these two groups, the differences in water and electrolyte excretion at rest in relation to the differences between cardiovascular and renal hemodynamic measurements in the two groups could not be evaluated.

During exercise. During exercise the averages of renal plasma flow and glomerular filtration rate in Group A were 216 ml. per min. per 1.73 m² and 71 ml. per min. per 1.73 m², respectively. Thus, in response to exercise in Group A the renal plasma flow decreased on the average 99 ml. per min. per 1.73 m² or 31 per cent and the glomerular filtration rate fell on the average 28 ml. per min. per 1.73 m² or 28 per cent. These decreases during exercise in Group A were highly significant statistically. In Group B during exercise the averages in the renal plasma flow and glomerular filtration rate dropped to 232 ml. per min. per 1.73 m² and 80 ml. per min. per 1.73 m², respectively. Thus, the mean decreases in renal plasma flow and glomerular filtration rate in response to exercise in the patients in Group B were considerably less than in Group A, the average renal plasma flow decreasing by 56 ml. per min. per 1.73 m² or 20 per cent and the glomerular filtration rate by 6 ml. per min. per 1.73 m² or 7 per cent. Although the fall in the renal plasma flow in response to exercise in Group A was not significantly greater than that in Group B, the decrease in glomerular filtration rate in Group A was significantly greater than the relatively unchanged response in Group B. The filtration fraction, elevated at rest in both Group A and B (averaging 34 and 33 per cent, respectively), increased only slightly during exercise in Group A but significantly in Group B. This latter change was consistent with the disproportionately greater reduction in the renal plasma flow than in the glomerular filtration rate during exercise.

In the Group A patients during exercise the decreases in the excretion of water (56 per cent), sodium (40 per cent), chloride (30 per cent), and potassium (27 per cent) were highly significant. In general, these reductions tended to parallel the decreases in the renal plasma flow and glomerular filtration rate induced by exercise. In two patients (A. J., F. A.) the renal hemodynamic measurements did not change but there was nevertheless a decrease in electrolyte excretion during exercise. In another patient J. C. there were significant changes in the renal hemodynamic measurements unassociated with changes in rate of electrolyte excretion. Two patients in Group A (C. J., C. S.) showed a significant decrease neither in renal hemodynamic function nor in excretion of electrolytes during exercise.

On the average, the Group B patients had less marked decreases in the excretion of water, sodium, chloride, and potassium during exercise. Of these measurements the reductions in sodium and potassium excretion were statistically significant. Only two of the patients (J. M., M. M.) had falls in both renal clearances (PAH and inulin) and in excretion of electrolytes and water during exercise. One patient (D. K.) showed no change in either renal clearances or excretory function. Another patient (M. G.) had a fall in the excretion of sodium, chloride, and potassium despite no reduction in the renal clearance measurements. Finally another patient (W. D.) showed a decrease in renal clearances without any appreciable change in the excretion of sodium and chloride. The magnitude of the decreases in the excretion of sodium, chloride, and potassium in response to exercise was not significantly different in Group A and Group B.

Recovery from exercise. In six of ten patients in Group A in whom exercise had produced depression of renal hemodynamic measurements, these functions had returned to the pre-exercise control values within 20 to 30 minutes of the recovery period. In the other four patients persistently low values of the glomerular filtration rate and/or renal plasma flow were observed during the entire recovery period of measurement which lasted for 26 to 45 minutes after the completion of exercise. The excretion of electrolytes,

[4] The values for renal plasma flow and glomerular filtration rate are lower than those reported by Fishman, Maxwell, Crowder, and Morales (9) in a similar group of patients with chronic pulmonary disease without congestive failure. These differences may be due to a greater incidence of clinically unrecognizable renal disease or arteriosclerotic changes of the kidneys in our group.

and usually of water, remained at a low level throughout the recovery phase not only in those patients who had a persistent lowering of the renal plasma flow and glomerular filtration rate but also in patients in whom the latter functions had shown prompt recovery. This persistent decrease in the excretion of water and electrolytes in most of the patients, therefore, appeared to be independent of changes in the renal clearance measurements (Figure 1).

In Group B the renal plasma flow and/or glomerular filtration rate had returned to the control value in all the patients in 20 to 30 minutes after the exercise period. However, the persistent reduction in the excretion of sodium and chloride in four patients (M. A., W. H., J. M., M. G.) during the recovery phase again suggested that the retention of these electrolytes was independent of any measurable alterations in renal plasma flow or glomerular filtration rate. Except in a few patients the excretion of water returned to the preexercise control level during the recovery period. Although the excretion of electrolytes tended to return more promptly to the control values in the Group B patients during the recovery phase, statistical analysis of the mean data showed no significant difference between Group A and Group B.

Two patients with so-called "high output" failure (M. S. with hypertensive cardiovascular disease and acute posthemorrhagic anemia, and H. H. with large systemic arteriovenous fistula) who were not included in the statistical analysis, also had abnormally low renal plasma flows and glomerular filtration rates at rest. Patient H. H. had the higher cardiac output and the more depressed renal plasma flow at rest. Both patients during exercise showed increases in intracardiac and pulmonary arterial pressures but had normal increases in cardiac output. With these cardiohemodynamic responses during exercise, however, there was slight or no change in the renal plasma flow and glomerular filtration rate. Patient M. S., who showed no significant change in either glomerular filtration rate or renal plasma flow during exercise, had a marked and persistent decrease in the excretion of water and sodium after exercise. Patient H. H. showed a transitory decrease in the excretion of sodium during exercise, but prior evidence of marked retention of sodium at

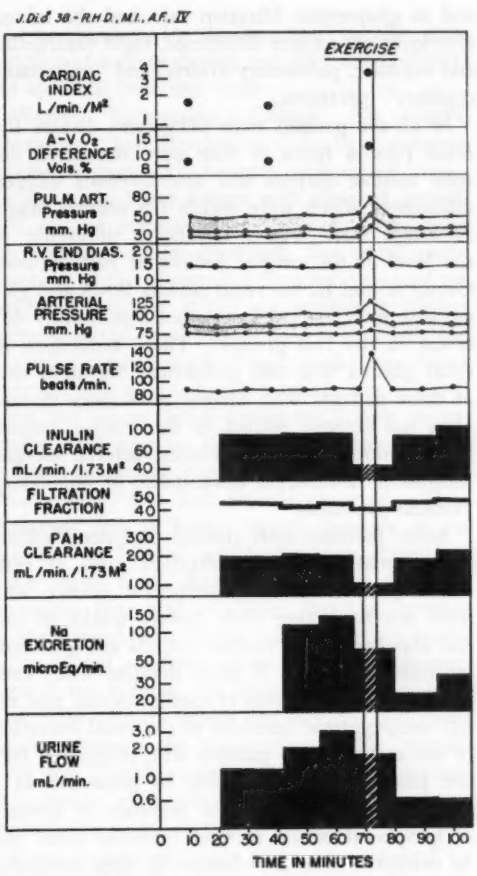
J.Di. 38-RHD, MI, AF, IV
EXERCISE
CARDIAC INDEX L./min./M²
A-V O₂ DIFFERENCE Vols. %
PULM ART. Pressure mm. Hg
R.V. END DIAS. Pressure mm. Hg
ARTERIAL PRESSURE mm. Hg
PULSE RATE beats/min.
INULIN CLEARANCE mL./min./1.73 M²
FILTRATION FRACTION
PAH CLEARANCE mL./min./1.73 M²
Na EXCRETION microEq/min.
URINE FLOW mL./min.
0 10 20 30 40 50 60 70 80 90 100
TIME IN MINUTES

FIG. 1. CHART OF THE CARDIOVASCULAR AND RENAL MEASUREMENTS AT REST AND DURING EXERCISE IN THE SUPINE POSITION IN PATIENT J. DI. WITH SEVERE MITRAL INSUFFICIENCY

rest in this patient made further reductions in the excretion of urinary sodium difficult to evaluate accurately.

DISCUSSION

In this group of patients no significant correlation was found between the cardiac output and the renal blood flow and glomerular filtration rate at rest. Statistical analysis of a larger group of thirty-eight patients with "low output" congestive failure studied in this laboratory likewise showed no absolute relationship between these functions. Also there was no consistent correlation between the degrees of abnormality in renal plasma flow

and in glomerular filtration rate and the mixed arteriovenous oxygen difference, right ventricular end diastolic, pulmonary arterial and "pulmonary capillary" pressures.

In all the patients with pulmonary disease the renal plasma flows at rest were decreased despite cardiac outputs and arteriovenous oxygen differences which were within the normal range. Although there was a significant difference in the level of the cardiac indices at rest between Group A and B, the renal plasma flows and glomerular filtration rates were not significantly different in the two groups. These reductions in renal plasma flow and glomerular filtration rate in these patients with chronic pulmonary disease were not directly related to the cardiac output, arteriovenous oxygen difference, arterial oxygen saturation, hematocrit, intracardiac or pulmonary vascular pressures.

Some patients with clinical peripheral edema had normal glomerular filtration rates at rest. Other patients without peripheral edema who were not restricting their dietary intake of salt had abnormally low cardiac outputs and glomerular filtration rates. A more detailed study concerning the relationship of cardiovascular and renal hemodynamic functions to the renal excretion of salt and water in patients with congestive failure has been done and will be presented in a separate report (10). The presence or absence of peripheral edema in these patients could not be predicted from the changes in their cardiodynamics or renal responses during or after exercise.

In the present study on the cardiac and renal responses to exercise the effects of postural factors have been eliminated. Numerous investigators have reported that exercise in normal subjects causes decreases in renal plasma flow and, to a lesser extent, in the glomerular filtration rate and that these falls are greater in the upright than in the supine position (1–5). The degree of depression of the renal clearance measurements has also been related roughly to the severity of the exercise (3). Merrill and Cargill (11) have found that the reduction in the renal plasma flow and glomerular filtration rate during exercise is greater in supine patients with congestive heart failure than in normal subjects. This greater decrease in the renal circulation has been considered to be related to an inadequate cardiac output in the cardiac patients during exercise. Exercise in the upright position in normal subjects and also in patients with congestive failure has been shown to produce persistent decreases in the excretion of water, sodium and usually of chloride (1–6). In general these persistent decreases appeared to be independent of changes in the glomerular filtration rate and therefore were attributed to increased tubular reabsorption on a hormonal basis. Sinclair-Smith, Kattus, Genest, and Newman suggest that the persistence of the depression of sodium excretion after exercising in the upright position is about the same in patients with congestive heart failure as in normal subjects (6).

In normal subjects exercising in the supine position for one-half hour by alternate straight leg-raising (resulting in a 3 to 9 fold increase in oxygen consumption), Hatcher, Huckabee, Epstein, and Wilkins (7) have observed a significant reduction in renal plasma flow and only a slight or no decrease in the glomerular filtration rate. The renal plasma flow usually remained depressed for the first 10 to 15-minute period after the completion of exercise and returned to the control values by the second or third recovery periods. A reduction in sodium excretion was sometimes observed during exercise, and this was usually associated with a decrease in glomerular filtration rate. Moreover, when the glomerular filtration rate and renal plasma flow recovered to control values following exercise, there was a prompt return of sodium excretion to the initial control levels. These studies suggest that although exercise may cause a decrease in the renal plasma flow and, to a lesser degree, glomerular filtration rate, exercise *per se* does not cause a persistent reduction in sodium and water excretion in normal healthy subjects. They also suggest that the greater tubular reabsorption of sodium and water and the larger decreases in glomerular filtration rate and renal plasma flow in patients exercising in the upright position is for the most part related to the posture itself. This view is supported by other studies (3, 4, 12–14) which have shown that the upright posture alone in normal subjects causes an increased renal tubular reabsorption of sodium and water and a decrease in renal plasma flow associated with slight or no fall in glomerular filtration rate.

Since in the present study the influence of postural factors on salt and water excretion have been eliminated, the renal responses to exercise may be compared to those observed by Hatcher, Huckabee, Epstein, and Wilkins. In patients with "low output" types of congestive failure the reductions in renal plasma flow and glomerular filtration rate were greater during exercise than in normal subjects. However, the renal hemodynamic responses during exercise in patients with chronic pulmonary disease resembled those of the normal subjects in showing a slight or insignificant reduction in the glomerular filtration rate. In general the return of the renal hemodynamic measurements to control values after a fall in response to exercise, in patients with any form of congestive failure, required a longer period of time than in normal subjects. Furthermore, the patients with "low output" forms of heart disease and those with chronic pulmonary disease had a more definite and greater decrease in sodium excretion during exercise than normal subjects. In the patients with "low output" congestive failure the reduction in sodium excretion usually paralleled the fall in the glomerular filtration rate while in the patients with chronic pulmonary disease, who had comparable changes in sodium excretion, this alteration in glomerular filtration rate was not so frequently observed. (Unfortunately, in two patients with the most severe cor pulmonale and congestive failure no measurements of the renal hemodynamics could be obtained during exercise.) A majority of patients in congestive failure had a more persistent reduction in sodium excretion after exercise than did the normal subjects. This persistent decrease in sodium excretion following exercise usually appeared to be independent of changes in glomerular filtration rate and was attributed to increased renal tubular reabsorption. The degree and duration of depression of the renal blood flow and glomerular filtration rate during and following exercise was not necessarily related to the degree and duration of the changes in cardiac output, mixed arteriovenous oxygen difference, arterial oxygen saturation, and the intracardiac, pulmonary arterial and renal venous pressures. Calculation of correlation coefficients also failed to reveal any correlation between the variation in the sodium excretion and the various cardiovascular responses. The mechanisms for the increased tubular reabsorption of sodium during and after exercise were not apparent from this study.

Werkö, Varnauskas, Eliasch, Ek, Bucht, Thomasson, and Bergström (15) have reported in clinical group IV patients with mitral stenosis and right heart failure with elevated peripheral venous pressure that although exercise in the supine position produced the usual slight lowering of the glomerular filtration rate and retention of sodium, it did not produce a further decrease of the renal plasma flow as it usually did in similar patients not in congestive failure. Since their patients with congestive failure also differed from the compensated group by failing to increase their cardiac outputs with exercise, the authors concluded that maximal depression in renal plasma flow had already occurred. They explained this as being "mainly because of the low cardiac output which did not allow any 'superfluous' renal blood flow." From these observations they also concluded that the decrease in renal blood flow during exercise is not necessarily related to further elevations of venous pressure in the kidney. In contrast, in our patients with "low output" failure and elevated peripheral venous pressure the low resting cardiac outputs were still capable of being increased in response to exercise while the low renal plasma flows showed the usual further reductions. This difference in responsiveness might be explained by the fact that in the study by Werkö and associates the amount of exercise performed caused an average elevation of about 70 per cent in oxygen consumption, while the more strenuous exercise employed in the present study resulted in about 150 per cent increase in oxygen consumption.

SUMMARY

Studies of renal functional responses to exercise in cardiac patients in the supine position showed (in contrast to those in normal subjects who have a transitory fall in renal plasma flow and a slight or no change in the glomerular filtration rate and excretion of sodium):

1. That Group A patients (with severe and usually decompensated valvular or non-valvular heart disease) had significant reductions in the renal plasma flow (31 per cent), glomerular fil-

tration rate (28 per cent), and excretion of sodium (40 per cent).

2. That Group B patients (with severe chronic pulmonary disease with or without congestive failure) had a fall in renal plasma flow (20 per cent), and a slight decrease in glomerular filtration rate (7 per cent), associated with a significant reduction in the excretion of sodium (20 per cent).

3. That the significantly greater reduction in glomerular filtration rate in Group A than in Group B patients was not associated with a greater reduction in sodium excretion.

4. That in the recovery phase there was a more rapid return of the renal hemodynamic measurements than of the excretion of sodium to the pre-exercise control values. This dissociation suggests that the retention of sodium was independent of any measurable alteration in glomerular filtration rate and probably resulted from a change in renal tubular activity.

5. That neither the magnitude nor the duration of the depression of the excretion of sodium was consistently related to the degree or duration of reduction in the glomerular filtration rate.

6. That no correlation could be determined between the degree or duration of reductions in renal blood flow, glomerular filtration rate, or the excretion of sodium and the degree of the cardiovascular responses.

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STUDIES IN STEROID METABOLISM. XXVII. A COMPARISON OF THE STEROID RESPONSE TO ACTH AND CORTISONE IN NORMAL YOUNG MEN[1]

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This study deals with the isolation and quantitative estimation of urinary ketosteroids following the administration of adrenocorticotrophic hormone (ACTH) and cortisone acetate to two normal young men, with appropriate control studies of their endogenous steroid excretion. The study was undertaken to obtain information on several aspects of endocrine physiology. The first of these was to provide an acceptable comparison for an investigation of the influence of stress upon the young male subject as measured by the output of steroid hormone metabolites. The second was to secure basic information relating to the nature of the adrenal response and the metabolic pathways utilized when the adrenal gland was stimulated by its trophic hormone as well as when the pituitary secretion was suppressed by the administration of large doses of exogenous steroid. The third was to obtain data after intravenously administered ACTH for comparison with the several studies in which much larger amounts of this hormone were administered by the intramuscular route. The fourth was to obtain evidence from related metabolic experiments on single individuals, serving as their own controls, to test the several generalizations that have been made concerning the metabolic fate of steroid hormones. While the design of the studies and the methods employed provide information upon these topics, it should be emphasized that in certain respects the amount of hormone administered has been in the pharmacologic range and the end products studied constitute only a portion of the total metabolites.

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EXPERIMENTAL

The two subjects studied were hospitalized on the Metabolic Unit of the Peter Bent Brigham Hospital during both experimental periods. Both subjects were 21 years of age. The details of the studies were as follows.

Subject C21. Following a nine-day control period (Period 1) a daily intravenous infusion of 20 mg. of ACTH (Armour) dissolved in 500 ml. of 5 per cent dextrose in water was administered. These infusions continued for a total of 3 consecutive three-day periods (Periods 2, 3, and 4). The duration of each infusion was 8 hours. Except for 2.7 gm. of KCl per day, no other medication was given. Blood pressure and body weight remained essentially constant during the experimental period. Twenty days following the last day of ACTH administration, he was re-admitted to the Metabolic Unit for a six-day control period (Period 5). Then 500 mg. per day of cortisone acetate (Merck) was administered orally, in divided dosage, for 2 consecutive three-day periods (Periods 6 and 7). An inadvertent omission of one dose (125 mg.) of cortisone acetate on the fifth day (Period 7) was compensated by increasing the dose to 625 mg. on the sixth and last day of this experimental period. He received 2.7 gm. per day of KCl during the period of cortisone administration. Blood pressure and body weight did not change during this portion of the study.

Subject M21. The same lot of ACTH was administered, and essentially the same program was followed with this subject, as with subject C21. However, urines from only eight days of the ACTH administration period were studied in M21. A small weight gain was recorded at the end of this period. Blood pressure did not change. Sixteen days after the end of the ACTH experiment, he was re-admitted for the cortisone study. Details of this experiment were similar to those in the study on subject C21. Blood pressure did not change at the end of cortisone administration, but again a slight weight gain was recorded.

Control urines were pooled. Urine collected during the period of ACTH and cortisone administration were combined into consecutive three-day pools (except for the two-day ACTH pool (Period 4) in the study on subject M21). The urinary steroid conjugates were hydrolyzed by a combination of methods employing enzymatic (beef liver β-glucuronidase), mild and vigorous acid hydrolysis (see Method D [1]). The neutral

TABLE I

Ketosteroid (KS) values by Zimmermann reaction and "formaldehydogenic steroid" (FS)† values on various fractions of enzyme and acid hydrolyzed urine during control, cortisone and ACTH periods of C21*

Fraction	Milligrams per 24 hours						
	Control	ACTH I.V. *20 mg./day*			Control	Cortisone	
A. Enzyme—neutral KS	16.9	25.8	31.3	35.6	17.3	35.6	37.7
B. Acid‡—neutral KS	12.9	12.9	17.3	15.5	14.2	11.3	10.1
C. Enzyme—water soluble ketonic KS	1.1	3.0	4.8	5.5	1.1	7.0	6.3
D. Enzyme—benzene soluble ketonic KS	13.7	23.0	25.6	27.1	15.3	21.1	18.6
E. Acid ketonic KS	7.9	9.1	12.5	11.6	9.8	6.6	6.5
F. Line D and E combined KS	21.6	32.1	38.1	38.7	25.1	27.7	25.1
G. "α" from F—KS	19.3	27.5	31.4	33.1	22.2	26.0	26.5
H. "β" from F—KS	1.6	2.8	4.0	4.3	3.1	2.2	0.9
I. "FS" of C	3.6	15.1	22.4	24.4	3.4	54.6	61.9
J. "FS" of non-ketonic from C	9.7	28.6	42.7	54.6	14.8	78.3	
K. "FS" of D	0.5	8.9	13.3	12.0	2.7	3.5	36.2

* KS = Ketosteroid in mg. equivalents of dehydroisoandrosterone.
† FS = Formaldehydogenic steroids in mg. equivalents of desoxycorticosterone.
‡ Acid hydrolysis was carried out on the combined acidic fraction from which the neutral ether soluble extract was obtained and the residual urine after incubation with the enzyme followed by ether extraction.

steroid extract obtained after enzymatic hydrolysis was partitioned between benzene and water according to the method of Mason (2). The benzene soluble fraction was then combined with the neutral steroid extract from the acid hydrolysis. The combined extract was then separated into "ketonic" and "non-ketonic" fractions and the ketonic portion was further separated into "α" and "β" ketosteroids (3). "Formaldehydogenic" steroids, using the method of Hollander, Di Mauro, and Pearson (4), as well as ketosteroids, by the method of Talbot, Butler, MacLachlan, and Jones (5), were measured on appropriate fractions. These values are recorded in Table I for Subject C and Table II for Subject M. Only the "α-ketosteroids" (line G, Tables I and II) were employed for the isolation of the individual steroids.

Separation of the ketosteroids was achieved by adsorption chromatography on silica gel and the components were identified by infrared spectrometry. The separation of urinary ketosteroid metabolites by adsorption chromatography on silica gel, although extensively used in the past in these laboratories, has not hitherto been described. The general procedure used was, in all respects, very similar to that employed for chromatography with alumina as the adsorbent except for the sequence of solvents used for elution. The ratio of adsorbent to ketosteroid (in terms of dehydroisoandrosterone as standard) was customarily 500:1. The material to be chromatographed was placed upon the previously prepared column in as small a volume as practical of a mixture of petroleum ether and approximately 0.5 per cent ethyl acetate. The column was then developed with small increments of ethyl acetate in petroleum ether. The acetates of androsterone and etiocholanolone were usually eluted with petroleum ether: ethyl acetate 93:7. Androstane-3,17-dione and etiocholane-3,17-dione were usually eluted with the same mix-

TABLE II

Ketosteroid (KS) values by Zimmermann reaction and "formaldehydogenic steroid" (FS)† values on various fractions of enzyme and acid hydrolyzed urine during control, cortisone and ACTH periods of M21*

Fraction	Milligrams per 24 hours						
	Control	ACTH I.V. *20 mg./day*			Control	Cortisone	
A. Enzyme—neutral KS	18.0	31.4	28.4	28.0	20.7	35.6	38.0
B. Acid‡—neutral KS	10.8	13.8	18.0	13.7	12.0	17.2	14.8
C. Enzyme—water soluble ketonic KS	1.3	3.0	3.6	3.8	1.6	5.9	5.0
D. Enzyme—benzene soluble ketonic KS	15.6	27.1	23.2	20.9	16.5	34.1	25.7
E. Acid ketonic KS	7.6	8.4	14.2	11.4	8.1	7.4	12.0
F. Line D and E combined KS	23.2	35.5	27.4	32.3	24.6	41.5	37.7
G. "α" from F—KS	23.5	32.3	35.2	28.9	22.1	38.7	36.8
H. "β" from F—KS	1.1	1.2	2.4	1.7	0.6	1.1	1.4
I. "FS" of C	2.8	14.1	13.5	17.4	3.2	44.0	45.5
J. "FS" of non-ketonic from C	11.2	33.5	37.7	55.0	25.7	60.1	70.5
K. "FS" of D	1.0	8.3	12.5	11.2	2.4	7.4	7.6

* KS = Ketosteroid in mg. equivalents of dehydroisoandrosterone.
† FS = Formaldehydogenic steroids in mg. equivalents of desoxycorticosterone.
‡ Acid hydrolysis was carried out on the combined acidic fraction from which the neutral ether soluble extract was obtained and the residual urine after incubation with the enzyme followed by ether extraction.

TABLE III

Subject C21—Ketosteroid response to intravenous ACTH and oral cortisone acetate after chromatographic separation of "α" ketosteroids (line G, Table I)

Compounds	Milligrams per 24 hours						
	Control	ACTH	ACTH	ACTH	Control	Cortisone	Cortisone
Total C_{19} 11-oxysteroids	2.8	5.3	8.9	8.0	2.6	12.0	16.3
Total C_{19} 11-desoxysteroids	12.2	20.4	19.8	17.9	13.5	9.5	6.4
3α-Hydroxyetiocholane-11,17-dione	1.1	3.2	1.4	1.1	1.0	9.3	10.4
3α, 11β-Dihydroxyetiocholane-17-one	—	tr.	2.1	2.8	tr.	tr.	1.7
3α, 11β-Dihydroxyandrostane-17-one	1.7	2.1	5.4	4.1	1.6	2.7	4.2
Etiocholanolone	6.0	8.4	8.4	7.4	5.9	4.1	3.6
Androsterone	6.2	12.0	11.4	10.5	7.6	5.4	2.8
Ratio Androsterone : Etiocholanolone	1.0	1.4	1.4	1.4	1.3	1.3	0.8

ture in the proportion 91 : 9. Androsterone is rather sharply removed from the chromatogram with 89.5 : 10.5, while a trail of this compound mixed with small amounts of etiocholanolone was usually seen when 89 : 11 was employed. Etiocholanolone was eluted with 88.5 : 11.5 and continued in the eluates up to the proportion of 87.5 : 12.5. 3α,17α-Dihydroxypregnane-20-one was the next substance recognized and was eluted with an ethyl acetate concentration of about 20 per cent. To this stage of the chromatogram the separation is excellent and in all respects far superior to that which can be obtained by the use of alumina as an adsorbent. The separation of steroids, in fact, was comparable to that obtained by the use of partition chromatography. For the more polar compounds, however, although superior to alumina, the silica gel chromatogram is decidedly inferior to the partition chromatogram. 3α,11β-Dihydroxyandrostane-17-one together with a very small trail of 17α-hydroxypregnanolone is eluted with petroleum ether-ethyl acetate 74 : 26. 3α,11β-Dihydroxyetiocholane-17-one follows immediately after this substance and is obtained in the eluates with 28 to 32 per cent ethyl acetate. 3α-Hydroxyetiocholane-11,17-dione is obtained immediately thereafter with 34 to 36 per cent ethyl acetate. A small amount of 3α,11β-dihydroxyetiocholane-17-one invariably accompanies the 3α-hydroxyetiocholane-11,17-dione. Recognition of the mixtures obtained was made by comparison of the infrared spectra with those of known mixtures of the pure compounds. With careful operation of the chromatogram a separation satisfactory for the purposes of these studies is easily achieved.

RESULTS

Quantitative values for isolated ketosteroids during both experimental periods for C21, are listed in Table III and for M21, in Table IV.

Subject C21

The control values for each of the individual compounds studied were typical of those observed in healthy young males (1). The sum of the C_{19} 11-desoxysteroids promptly increased, during the first period of ACTH administration. Then, despite the continued administration of ACTH, these compounds remained at essentially constant, though increased levels. Both etiocholanolone and androsterone were augmented, the latter somewhat more than the former. The increase in levels of C_{19} 11-oxygenated steroids was relatively of greater magnitude than that of the C_{19} 11-desoxy compounds. This appears to be part of

TABLE IV

Subject M21—Ketosteroid response to intravenous ACTH and oral cortisone acetate after chromatographic separation of "α" ketosteroids (line G, Table II)

Compounds	Milligrams per 24 hours						
	Control	ACTH	ACTH	ACTH	Control	Cortisone	Cortisone
Total C_{19} 11-oxysteroids	2.8	8.4	11.1	8.1	3.7	18.6	15.3
Total C_{19} 11-desoxysteroids	14.7	16.8	19.1	15.0	12.2	5.7	4.5
3α-Hydroxyetiocholane-11,17-dione	0.8	3.1	2.0	2.0	1.5	12.7	9.3
3α, 11β-Dihydroxyetiocholane-17-one	0.4	1.6	4.8	3.1	0.6	4.3	2.3
3α, 11β-Dihydroxyandrostane-17-one	1.6	3.7	4.3	3.0	1.6	1.6	3.7
Etiocholanolone	7.6	9.2	10.9	8.2	6.3	3.2	2.7
Androsterone	7.1	7.6	8.2	6.8	5.9	2.5	1.8
Ratio Androsterone : Etiocholanolone	0.9	0.8	0.8	0.8	0.9	0.8	0.7

the normal characteristic pattern of adrenal response to ACTH (6). All components of this group of steroids, 3α-hydroxyetiocholane-11,17-dione, 3α,11β-dihydroxyandrostane-17-one, and 3α,11β-dihydroxyetiocholane-17-one shared initially in this increase, but during the later periods, the C_{19} 11-hydroxylated compounds predominated.

Steroid levels during the second control period were, as expected (7), essentially the same as during the first control. During cortisone administration, the C_{19} 11-desoxysteroids decreased in stepwise fashion, with etiocholanolone decreasing somewhat less than androsterone. As expected, levels of C_{19} 11-oxygenated steroids increased markedly with 3α-hydroxyetiocholane-11, 17-dione predominant. Small increases in 3α,11β-dihydroxyetiocholane-17-one, and still smaller but distinct increases of 3α,11β-dihydroxyandrostane-17-one were found.

Subject M21

Essentially similar metabolic results were observed in this subject. Under ACTH stimulation, levels of both 11-oxygenated and 11-desoxysteroids increased; the former, as expected, to a relatively greater extent. The increase in 11-desoxysteroids in this subject, was not as pronounced, nor as sustained as that in C21 and androsterone excretion was not measurably increased. The qualitative and quantitative pattern of C_{19} 11-oxygenated steroids was essentially similar to that observed in C21. Both etiocholanolone and androsterone decreased during cortisone administration. The increase in C_{19} 11-oxygenated steroids during cortisone administration was similar but not as pronounced as that observed in C21.

DISCUSSION

The consistent rise in output of 11-desoxy-17-ketosteroids following the administration of ACTH to these and other (6) normal men was the most striking, though perhaps not unexpected, alteration in urinary ketosteroids observed. Although the two compounds, androsterone and etiocholanolone, represent the major metabolites of testosterone in man (8–10), the fact that they are consistently present in the urine of normal women (8), are identified in the urine of castrate males and females (11), and are excreted in large amounts in frank adrenal disease (12) indicates the existence of an adrenocortical precursor as well. While the absolute amounts of these two substances increased, the ratio of one to the other throughout the interval of stimulation by ACTH remained essentially unaltered (Tables III and IV). This relatively constant ratio was maintained during the period of cortisone administration with the exception of the last period in C21. This finding indicates that exogenous cortisone equally suppressed the production of the precursors of both metabolites. A simple and not unreasonable interpretation would be that the maintenance of a constant ratio during cortisone administration reflected suppression of a single precursor of both compounds. It is likely that this precursor was of adrenal origin, although depression of some testicular or pituitary hormone by the large dose of cortisone cannot be excluded. Since both androstane and etiocholane isomers were increased during ACTH administration it would be reasonable to assume that a C_{19} steroid such as testosterone, Δ^4-androstene-3,17-dione or dehydroisoandrosterone was secreted by the adrenal gland. The magnitude of the increase or decrease of the 11-desoxy metabolites is consistent with this view, since each of these potential precursors is converted to both urinary 17-ketosteroids. The amount of *hormone* secreted (or suppressed) would approximate twice the increment (or decrement) found as metabolites in the urine (10) on the presumption that testosterone was the precursor. The conversion of C_{21} 17-hydroxy precursors, such as 17α-hydroxyprogesterone or Reichstein's "Substance S" to 17-ketosteroids is an inefficient biochemical process as evidenced by the small yield of C_{19} 17-ketosteroids (13, 14); the same is true for cortisone, exemplified in the present studies, as well as in others in which both isotopic and unlabelled precursors were employed (15–17). It seems logical, therefore, to defer consideration of a C_{21} steroid as a potential precursor and to focus attention upon more proximate C_{19} steroids which could normally be present in the internal secretions from the adrenal gland and would be increased under intense stimulation by ACTH.

The choice of a single compound from the more likely C_{19} precursors mentioned is difficult but

there is indirect evidence that points to a plausible selection. It is clear that in certain conditions such as adrenal hyperplasia of the virilizing type and in less frank manifestations of adrenal hypersecretion such as excessive hirsutism in women (18, 19) the adrenal gland can produce a physiologically active androgen. This is apparent not only from the overt manifestations seen in the several clinical states but is evident from the finding of elevated quantities of the metabolites of androgens (*e.g.*, androsterone and etiocholanolone) in the urine. Of the three compounds under discussion, testosterone and Δ^4-androstene-3,17-dione are the more effective in this respect. Moreover, while these two compounds can be readily metabolized to androsterone and etiocholanolone by man, they are not transformed to dehydroisoandrosterone (9, 20, 21). Since the "β" ketosteroid fractions from the urine of these two subjects showed only minor increases during ACTH administration, and since the excretion of dehydroisoandrosterone can be so greatly increased in abnormal adrenal hyperactivity (12), it would seem unlikely that dehydroisoandrosterone was, under physiologic circumstances, the major adrenal precursor of these two metabolites. As between testosterone and Δ^4-androstene-3,17-dione, the evidence favors the latter since this compound has been isolated from beef adrenal extracts by von Euw and Reichstein (22) and evidence for its presence in cow adrenal perfusates has been adduced by Bloch, Dorfman, and Pincus (23). Very recently Bloch, Benirschke, and Dorfman (24) have obtained evidence for the presence of this compound in a human fetal adrenal and the adrenal of a newborn male pseudohermaphrodite. At present there is insufficient evidence available on the adrenal hormones in man to make a definitive statement about the chemical nature of the androgen which serves as the precursor of the metabolites androsterone and etiocholanolone. It is clearly necessary to study the 11-desoxysteroids from the human adrenal and we have initiated studies of this problem. As a working hypothesis, however, the suggestion can be advanced that either Δ^4-androstene-3,17-dione or testosterone would account well for the findings demonstrated in this investigation.

Following the administration of cortisone the urinary C_{19} 11-oxygenated ketosteroids have been studied in detail (15) and the results in subject C21 and M21 confirm the general conclusions drawn from these previous data. In particular, it is evident that 3α,11β-dihydroxyandrostane-17-one is a metabolite, though in small amount, of cortisone, and presumably hydrocortisone. It is clear from previous work that the 11-keto and 11β-hydroxy functional groups are metabolically interconvertible; the degree and direction of this oxidation-reduction may reflect a variety of factors, the significance of which remains for further clarification. Thus in the subject studied by Burstein, Savard, and Dorfman (15), the major C_{19} 17-ketosteroid metabolite of cortisone was 3α,11β-dihydroxyetiocholane-17-one, while in our two subjects, the major 17-ketosteroid metabolite was 3α-hydroxyetiocholane-11,17-dione. The predominant conversion of adrenosterone to 3α,11β-dihydroxyandrostane-17-one (25), rather than to the 11-keto analog, poses similar problems of interpretation.

Dorfman (26) has reached the tentative conclusion that 3α,11β-dihydroxyandrostane-17-one in the urine reflects, to a considerable extent, the metabolic transformation of a C_{19} 11-oxygenated precursor. Thus, this metabolite could arise from a C_{21} adrenal hormone such as hydrocortisone by a metabolic alteration that removed the two carbon side-chain prior to any change in the α,β unsaturated ketone system in Ring A or directly from a C_{19} precursor such as adrenosterone or 11β-hydroxy-Δ^4-androstene-3,17-dione. Since the latter two products are undoubtedly secreted by the adrenal gland but do not serve the vital functions of hydrocortisone, their metabolites may reflect only indirectly the extent of the adrenal response to stress. With these possibilities in view, it may be more significant to direct attention to the individual ketosteroids rather than to consider all these metabolites as a group. A more accurate indication of "adrenal response" with respect to metabolically active steroids of the hydrocortisone type would be the urinary levels of "non-ketonic" C_{21} products of hormone metabolism such as pregnane-3α,11β,17α,20α,21-pentol and related isomers (27) since these compounds account for a higher proportion of the total metabolites than do the C_{19} ketosteroids.

The increase in levels of 3α,11β-dihydroxyandrostane-17-one and 3α,11β-dihydroxyetiocholane-17-one during the administration of ACTH

approached the increase in these compounds following cortisone administration. After an initial increase, the levels of 3α-hydroxyetiocholane-11,17-dione during ACTH administration decreased steadily to control values. The concurrent increase in levels of other 11-oxygenated metabolites makes it unlikely that this represents technical error or loss. A similar response was noted in a subject treated with ACTH intramuscularly (6). The results in these subjects therefore parallel those reported earlier. A point of difference from the earlier studies of the effect of ACTH in young men is found in the relatively minor increase in the "β" ketosteroids of the two subjects of the present investigation. Whereas one of the previous subjects showed an increase in "β" ketosteroids to a level of 13 mg. per day at the peak value, no such relative or absolute change was apparent with these two men. In our experience, the alterations in the "β" fraction may be erratic and the changes observed cannot always be correlated with physiological or biochemical processes. In general terms, however, the present study agrees well with the prior report, when consideration is given to the several methodological changes as well as the different route of ACTH administration. In addition it may be noted that the quality of the ACTH available for the present study was probably superior to that used in the earlier investigations.

A rough comparison of the dose-response relationships after ACTH and cortisone acetate administration can be made from these, and previous data (6). From the increase in excretion of C_{19} 11-oxygenated metabolites during the two experimental periods, 20 mg. of ACTH administered as in this study is approximately equal to 150 to 250 mg. of cortisone acetate orally. On the same basis 20 mg. of ACTH intravenously administered produced about the same response as 100 mg. of ACTH administered by the intramuscular route. Quantitative relationships of this nature are approximations, however, since the limitations of these comparisons are evident. Alterations in the rate of absorption of administered hormones from the intramuscular depot or the gastrointestinal tract, variations in the degree of hormonal alteration and the unknown influences of biologic variations in experimental subjects may seriously influence not only quantitative relationships, but also qualitative patterns of metabolism as well.

SUMMARY

The qualitative and quantitative pattern of ketosteroid response has been studied in two normal young men treated experimentally with large doses of oral cortisone acetate and intravenous ACTH. Conclusions have been drawn concerning the metabolic pathways utilized when large doses of adrenal hormones are provided from exogenous, as compared with endogenous sources. In the latter situation the ketosteroid response indicates the existence of a C_{19} 11-desoxy substance of adrenal origin. This hormonal substance, we conclude from a consideration of available data may represent adrenocortical production of testosterone or a chemically closely related compound and the metabolites may thus be a measure of the primary adrenal androgen.

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STUDIES IN STEROID METABOLISM. XXVIII. THE α-KETOSTEROID EXCRETION PATTERN IN NORMAL FEMALES AND THE RESPONSE TO ACTH[1]

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An examination of the urinary α-ketosteroids has been made in normal women in order to provide information about this important group of hormone metabolites. These data establish limits of values for the major components of this group of steroids and thus provide not only a suitable comparison for studies in abnormal or altered hormonal physiology, but offer information about possible sex differences in the composition of these hormonal metabolites as well. The qualitative and quantitative α-ketosteroid excretion pattern of a series of normal males has been reported earlier from this laboratory (1).

Two of the pre-menopausal women were studied at successive intervals throughout the menstrual cycle. The results provide evidence that the precursors of the major urinary neutral ketosteroids in normal women are secreted independently of this pituitary-ovarian rhythm. In addition, two other normal young women were studied during control periods as well as during successive periods while under vigorous stimulation with adrenocorticotrophic hormone (ACTH). The results provide information on the character of this adrenal response in women, and are thus helpful in the interpretation of abnormal hormonal situations, an example of which is described in a recent study (2).

The development of methods for more satisfactory hydrolysis of urinary steroid conjugates, improvement in the techniques of separation, particularly partition column and paper chromatography and increased facility in the recognition of individual compounds by infrared spectrophotometry afford a high degree of assurance about the reproducibility of the data. Thus, while the number of individuals studied is small, physiologic interpretation and projection to the normal female population is permissible with a high degree of probability.

EXPERIMENTAL

Eight normal healthy white females, described by initial and age were studied; M22, W25, S33, F38, B43, N47 were pre-menopausal and G53, W62 were post-menopausal. Subject S33 was studied during 3 consecutive periods throughout a single menstrual cycle; subject F38 was studied similarly during 4 consecutive periods. The steroid values reported for these two subjects represent the averages of these replicate steroid patterns: the reproducibility of these patterns was excellent. Subject M22 was studied during 6 consecutive periods. Following a six-day control period (Period 1) 80 units of ACTH (Armour Laboratories H. P. Acthar® Gel) were administered daily in a single dose intramuscularly for 3 consecutive three-day periods (Periods 2, 3, and 4). Following this treatment, two consecutive post control periods, totalling six days (Periods 5 and 6) were studied.

Subject W25 was studied similarly employing the same dose of ACTH gel. The ACTH period was, however, slightly shorter (see Periods 2, 3 and 4, Table IV). Both subjects became slightly edematous towards the end of the period of ACTH administration; no other untoward effects were noted. Diuresis occurred promptly following the termination of treatment.

All subjects were ambulatory. Complete urine collections were obtained and were checked by means of daily creatinine determinations. Urines were refrigerated until processed, which was usually begun one or two days following the completion of each three-day period. Enzymatic and acid hydrolysis of the urinary steroid conjugates was accomplished according to the following method, which for reference purposes (see Methods A, B, C, and D [1]) is called Method E. The details are as follows: *Method E. Stage 1.* The urine was adjusted to pH 5 with sulfuric acid; 10 per cent by volume of sodium acetate buffer at pH 5 and 300,000 units per liter of beef liver β-glucuronidase were added. The solu-

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[2] Research Fellow of the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

TABLE I

The urinary steroid excretion pattern in normal females

Compounds	Milligrams per 24 hours							
Total C_{19} 11-oxysteroids	1.5	1.6	1.6	1.1	1.0	1.6	2.4	1.4
Total C_{19} 11-desoxysteroids	5.7	4.7	3.6	2.3	1.6	3.1	2.3	0.4
3α-Hydroxyetiocholane-11,17-dione	0.6	0.6	0.6	0.3	0.4	0.5	0.7	0.5
3α, 11β-Dihydroxyetiocholane-17-one	0.5	0.4	0.5	0.3	0.3	0.6	1.1	0.4
3α, 11β-Dihydroxyandrostane-17-one	0.4	0.6	0.5	0.5	0.3	0.5	0.6	0.5
Etiocholanolone	3.2	3.2	2.2	1.4	0.9	1.7	1.6	0.2
Androsterone	2.5	1.5	1.4	0.9	0.7	1.4	0.7	0.2
β Ketonic KS	0.5	0.3	1.2	0.1	0.2	0.1	1.3	0.02
α Ketonic KS	9.5	7.2	10.4	6.1	5.6	6.3	9.1	3.2
Ketonic KS	10.0	9.1	12.5	7.2	5.7	6.9	11.5	3.5
Crude KS	13.7	11.7	18.6	13.4	11.1	9.6	29.5	9.6
Collection days	6	6	*	*	20	10	10	9
Patient—Age	M22	W25	S33	F38	B43	N47	G53	W62

* Average of values of replicate patterns.

tion was incubated at 37° for 120 hours, then adjusted to pH 1 with sulfuric acid and extracted with ether for 48 hours. The "crude neutral fraction" was obtained by extracting this ether solution with 2 N sodium hydroxide, followed by washes with 5 per cent sodium chloride solution until neutral.

Stage 2. The alkali soluble material and the sodium chloride washes were combined with the spent urine from Stage 1, and adjusted to acidity equivalent to 1 N sulfuric acid. This solution was then extracted for 72 hours with ether. A second "crude neutral fraction" was obtained from the ether extract as in Stage 1.

These two "crude neutral fractions" were individually separated into ketonic and non-ketonic fractions by means of Girard's "Reagent T," and into α and β-ketosteroid subfractions by precipitation with digitonin. These methods have been described in detail (3). The α-ketosteroid fractions were separately chromatographed.

In the majority of instances separation of the individual steroids was accomplished both by column partition chromatography (4), as well as by paper chromatography employing the systems of Burton, Zaffaroni, and Keutmann (5) and the modifications applicable to the less polar ketosteroids (6). For the partition columns, silica gel containing ethanol was used as the stationary phase and methylene chloride containing small amounts of ethanol was the mobile phase (4). Appropriate portions of each neutral extract were separated by both procedures and it was demonstrated that if the proper precautions were exercised, the agreement of the two methods was excellent. As a consequence, in the later portions of the study the more rapid paper chromatography was employed exclusively; wherever advisable, the identity of individual components was established by means of infrared spectrometry. The material from Stage 2 was separated by paper chromatography only. The quantitative analysis of the separated steroids was done by the Zimmermann reaction (as modified by

TABLE II

Replicate steroid pattern in normal females

Compounds	Milligrams per 24 hours						
Total C_{19} 11-oxysteroids	1.3	1.9	1.7	0.7	1.0	1.1	1.2
Total C_{19} 11-desoxysteroids	3.7	3.7	3.6	2.5	2.6	2.0	2.2
3α-Hydroxyetiocholane-11,17-dione	0.4	0.7	0.7	0.2	0.3	0.2	0.4
3α, 11β-Dihydroxyetiocholane-17-one	0.5	0.6	0.4	0.2	0.3	0.2	0.4
3α, 11β-Dihydroxyandrostane-17-one	0.4	0.6	0.6	0.3	0.4	0.7	0.4
Etiocholanolone	2.2	2.2	2.3	1.4	1.6	1.2	1.4
Androsterone	1.5	1.5	1.3	1.1	1.0	0.8	0.8
β Ketonic KS	2.0	1.0	0.8	0.1	0.1	0.1	0.1
α Ketonic KS	9.8	10.7	10.5	6.3	5.4	6.6	6.0
Ketonic KS	12.9	12.3	12.3	7.5	7.2	7.8	6.4
Crude KS	23.9	16.6	16.8	12.8	13.6	13.9	13.4
Collection days	6	9	8	6	6	3	6
Period No.	1–2	3	4	1–2	3–4	5	6–7
		S33			F38		

TABLE III

The ketosteroid excretion of a girl before, during and after ACTH—Subject M22

Compounds	Milligrams per 24 hours					
Total C_{19} 11-oxysteroids	1.5	5.3	4.9	6.2	2.6	1.5
Total C_{19} 11-desoxysteroids	5.7	11.4	13.5	15.4	6.2	3.4
3α-Hydroxyetiocholane-11,17-dione	0.6	1.6	1.8	2.0	1.0	0.5
3α, 11β-Dihydroxyetiocholane-17-one	0.5	2.4	2.3	2.8	1.1	0.4
3α, 11β-Dihydroxyandrostane-17-one	0.4	1.3	0.8	1.4	0.5	0.6
Etiocholanolone	3.2	7.6	9.4	10.2	4.2	2.2
Androsterone	2.5	3.8	4.1	5.2	2.0	1.2
Dehydroisoandrosterone	0.4	1.0	3.4	6.2	0.4	tr.
β Ketonic KS	0.5	1.9	5.8	8.0	0.9	0.2
α Ketonic KS	9.5	17.8	21.3	24.2	11.5	6.4
Ketonic KS	10.0	22.2	28.0	36.9	14.3	7.6
Crude KS	13.7	26.3	30.7	37.8	18.1	11.6
Collection days	6	3	3	3	2	4
Period No.	1	2	3	4	5	6
Therapy	(Control)		(ACTH Periods)		(Post Control)	

Callow, Callow, Emmens, and Stroud (7, 8) and Talbot, Butler, MacLachlan, and Jones (9). Appropriate factors were used to correct the color values from mg. of dehydroisoandrosterone, used as standard, to mg. of the actual compound determined in the chromatographic fraction, since ketosteroids differ somewhat in chromogenic value (10).

RESULTS

The quantitative data on the α-ketonic steroid excretion are given in Tables I, II, III, and IV. Subjects are described by initial and age. Table I lists these data for the eight normal subjects; values are reported for the crude neutral as well as the various fractions obtained prior to separation of the individual components. As noted earlier (1), the high values for crude extracts are the result of relatively complete extraction of ketones, non-ketones and non-specific chromogens. The values for the ketonic fraction are a closer approximation of the sum of the individual components.

In addition to the compounds reported in the Tables, a number of other ketosteroids are present in small amounts in the urine of normal women. Among the compounds always found in the "α-ketosteroid" fraction are: etiocholane-3,17-dione and androstane-3,17-dione, 3α-hydroxyandrostane-11,17-dione and 3α,17α-dihydroxypregnane-

TABLE IV

The ketosteroid excretion of a girl before, during and after ACTH—Subject W25

Compounds	Milligrams per 24 hours					
Total C_{19} 11-oxysteroids	1.6	2.5	6.1	11.8	4.2	1.5
Total C_{19} 11-desoxysteroids	4.7	8.8	11.4	18.1	7.7	3.1
3α-Hydroxyetiocholane-11,17-dione	0.6	0.6	1.4	3.0	1.2	0.4
3α, 11β-Dihydroxyetiocholane-17-one	0.4	1.1	2.9	5.5	2.4	0.6
3α, 11β-Dihydroxyandrostane-17-one	0.6	0.8	1.8	3.5	0.6	0.5
Etiocholanolone	3.2	6.1	7.7	12.7	5.3	2.1
Androsterone	1.5	2.7	3.7	5.4	2.4	1.0
Dehydroisoandrosterone	0.1	0.4	0.3	1.5	0.6	tr.
β Ketonic KS	0.3	0.8	1.1	2.4	0.9	—
α Ketonic KS	7.2	15.6	21.4	40.3	17.7	—
Ketonic KS	9.1	16.5	20.7	42.5	18.4	7.6
Crude KS	11.7	20.2	25.3	45.0	24.7	13.7
Collection days	6	2	2	3	3	3
Period No.	1	2	3	4	5	6
Therapy	(Control)		(ACTH Periods)		(Post Control)	

20-one;[3] small amounts of 3β-hydroxyetiocholane-17-one are usually found in the α-ketosteroid fraction and occasionally in the β-ketosteroid fraction. Usually, but not invariably, pregnanolone was present and 11β-hydroxyandrostane-3,17-dione was sometimes detected in trace amount. If the small β-ketosteroid fraction is separated into its components, dehydroisoandrosterone is invariably the major compound present, and isoandrosterone accompanies this compound. In addition 7-keto-dehydroisoandrosterone is usually found. Quantitative values for these substances have not been included since their contribution to the total is small and separation is often incomplete. It is nevertheless important to note that these, as well as small amounts of other as yet unidentified compounds, probably steroids, are normally present in the ketosteroid fraction.

All three 11-oxygenated steroids (Table I), *i.e.*, 3α-hydroxyetiocholane-11,17-dione, 3α,11β-dihydroxyandrostane-17-one, and 3α,11β-dihydroxyetiocholane-17-one were found in approximately equivalent amount in each individual studied and there was relatively little variation from subject to subject. The 11-desoxysteroids, *i.e.*, androsterone and etiocholanolone varied within much wider limits among the different individuals. Etiocholanolone was the major component and consequently exhibited the greatest fluctuation. The significance of these changes will be considered in the discussion.

Table II records the quantitative values for individual steroids and the groups from which these were derived, for two normal women during consecutive portions of the menstrual cycle in each subject. Urine collections commenced the first day following the menstrual period and ended the day preceding the onset of the succeeding flow. Thus in the strict sense the whole cycle was not studied. The constancy of the steroid excretion pattern over the intervals, examined, however, provides evidence that there is no marked variation or rhythm in the precursors of the metabolites of the neutral hormones, as distinct from the phenolic ovarian secretions.

Tables III and IV contain comparable data on the ketosteroid excretion of two young girls before, during and after treatment with ACTH. The steroid response to this pituitary hormone was in general terms similar in both subjects. 3α,11β-Dihydroxyetiocholane-17-one showed the greatest relative as well as absolute increase among the 11-oxygenated metabolites. All the 11-oxygenated steroids however, were at elevated levels during ACTH stimulation. The 11-desoxysteroids were similarly increased in both subjects. There was a slightly greater rise in etiocholanolone than in androsterone but the proportion of these two compounds did not alter appreciably throughout the experiment in either subject.

In the periods after ACTH the 11-oxygenated steroids quickly returned to normal levels in both girls. The decline in excretion of 11-desoxysteroids was equally prompt and it is of some interest to note that both subjects exhibited a similar pattern. This is evident in Period 6 when, in both girls, the 11-oxygenated metabolites, within experimental error, were at control levels. Despite this fact, the 11-desoxysteroids were appreciably lower than at any time during the study. The fact that the same result was obtained in the two girls, combined with the very considerable confidence with which these compounds can be separated and measured, lends a special significance to the observation despite the lack of statistical numbers and in the face of only a small change in absolute amount of metabolite.

DISCUSSION

A consideration of the chemical structure of these steroid metabolites and their probable hor-

[3] The prior failure to recognize 3α,17α-dihydroxypregnane-20-one as a normal constituent of most urines (11) was the result of a combination of methodological difficulties. Upon vigorous acid hydrolysis, the compound to a very considerable extent is converted to a D-homosteroid. When pure, 3α,17α-dihydroxypregnane-20-one is almost completely insoluble in carbon disulfide and soluble with difficulty in chloroform, so that recognition by infrared spectrometry was hampered. Although the substance was readily recognized in frank adrenal abnormalities where the amount present was relatively large, small amounts escape detection by the means available in the earlier studies. The use of more satisfactory hydrolysis and more efficient separation procedures, combined with greater familiarity of the limitations of infrared spectrometry for this special problem, facilitated the recognition of small amounts of the substance. A method for the quantitative estimation of 3α,17α-dihydroxypregnane-20-one has been recently developed in these laboratories and will be published in the near future.

monal precursors, makes it likely that these data define certain parameters of adrenocortical function and steroid hormone metabolism in normal women. These functions can be chemically categorized with respect to hormonal precursors with or without the oxygen function at carbon-11 of the steroid nucleus. It is presumed that all the ketosteroids measured in these studies reflect primarily adrenocortical hormones and that the 11-oxygenated steroids represent for the most part end products of hydrocortisone metabolism. The precursors of the 11-desoxysteroids are less well defined and interpretation of their function is more speculative.

As defined by these criteria, the results presented indicate that the synthesis, secretion and metabolism of hydrocortisone in normal women fluctuate only within narrow physiological limits. This is in contrast with the wide variation in 11-oxygenated steroids reported earlier for normal males (1). That this represents a real sex difference is problematical. It must be remembered that the studies with normal males were conducted over an extended interval of time during which a number of hydrolytic procedures was employed and while the methods of separation and recognition were constantly being improved. The constancy of the values for 11-oxygenated ketosteroids in this present study may reflect the success of these methodologic advances. While there are variations among the individual subjects, as would be expected on purely physiological grounds, in only one instance (G53; 3α,11β-dihydroxyetiocholane-17-one) was the variation within the group as great as four-fold for a single compound. The variation in excretion of 3α-hydroxyetiocholane-11,17-dione from subject to subject was only about two-fold in the most widely divergent instances and the amount of 3α,11β-dihydroxyandrostane-17-one measured was even more constant. Nevertheless an interesting feature emerges from these data. The mean value for C_{19} 11-oxygenated steroids in men was approximately 2.0 mg. while the corresponding figure for the normal females examined in this study was 1.5 mg. The methodological difficulties in the earlier studies, especially the failure to identify 3α,11β-dihydroxyetiocholane-17-one as a normal and sometimes major component of the C_{19} 11-oxygenated ketosteroids, would indicate that the mean values reported for these compounds in males were slightly lower than the true values. It would appear, then, that as a general rule, adrenal function, as defined by these criteria, is at a lower homeostatic level in the female.

Over the age range studied, adrenal function in women is independent of chronological age, as measured by the three C_{19} 11-oxygenated steroids that comprise the major constituents of this group of adrenal metabolites. This conclusion is in agreement with that reported in the normal male series and would lend support to the suggestion of a sex difference in the steady state level of adrenocortical activity. It would be of considerable interest to establish the validity of this conclusion in an extended series by a study of the more highly oxygenated metabolites of hydrocortisone, such as the cortols and cortolones (11) since these appear to represent a larger portion of the total metabolites. Efforts in this direction have already been initiated in this laboratory.

The C_{19} 11-desoxy ketosteroids in women and in men, as a general rule appear to diminish with age although, as anticipated, there are exceptions to the general trend. The precursors of these products, then, are not restricted to the narrow physiologic limits characteristic of the 11-oxygenated hormones. It seems unlikely that the decrease in 11-desoxysteroids of women with age represents a decline in the ovarian contribution to these metabolites since appropriate precursors have never been demonstrated in the gland. One must, therefore, conclude that there is a selective diminution in certain adrenocortical steroid synthesis, possibly androgen from the nature of the metabolites, that occurs with increasing chronologic age. This same process, inferentially, is operative in the male as well. These findings clearly require confirmation and elaboration with a larger group of subjects since the theoretical and practical import is apparent.

It is evident from the data presented in Table II that the secretion and metabolic transformation of the hormonal precursors of the C_{19} α-ketosteroid metabolites measured is not subject to marked fluctuation during the examined intervals of a menstrual cycle. These results do not preclude some alteration during active menstrual flow. With this reservation, the conclusion is of some importance in that it facilitates comparison of

data from random collections as well as between pre- and post-menopausal subjects with respect to the metabolites studied. In addition, it is of value for the further study of the sex difference suggested by the results of this report and extension of these findings to a comparison of normal and diseased subjects. While it may be desirable to study this problem more intensively with a view to daily variations that may be obscured in the longer term studies, these aspects must await the development of more rapid and easily applied methods.

The steroid response to ACTH in the normal females studied was characterized by a prompt and progressive increase in all the compounds measured. The magnitude of this response testifies to the more effective adrenocortical stimulation induced by ACTH gel. The percentage increase of 11-oxygenated steroids was greater than the percentage of 11-desoxy ketosteroids. Notwithstanding, the amount of 11-oxygenated steroids did not in any period exceed in absolute terms the total amount of 11-desoxysteroids in either subjects. This same pattern of response was manifest in both female subjects despite an almost 50 per cent difference in the rise in steroid excretion at the peak level.

The urinary β-ketosteroids, with dehydroisoandrosterone as the principal component, increased markedly during ACTH administration in both subjects. In fact, the increase in dehydroisoandrosterone output was proportionately the greatest of any of the compounds measured, *i.e.*, at the peak level, about 15-fold that found during the control period of either subject. Despite the marked rise, it should be noted that the amount of this β-ketosteroid did not parallel the response to ACTH as determined by either the 11-oxygenated or 11-desoxy α-ketosteroids. Thus, while both dehydroisoandrosterone and "dehydroisoandrosterone like substances" increase during adrenal stimulation with ACTH (12–14), it can be suggested from the disparity in the quantitative response of α and β ketosteroids that different precursor-product mechanisms may be involved in the elaboration and excretion of these various compounds.

The inference that the substantial rise in 11-desoxysteroids, is the reflection of a sensitive independent pathway of synthesis for an adrenal androgen, is an attractive interpretation of the results. In certain circumstances pathological overactivity of this synthetic pathway may have a clear clinical expression as in certain forms of hirsutism (2). It is interesting to note that within the confines of this increase the proportion of etiocholanolone to androsterone did not change markedly. Thus, although a C-21 precursor may have contributed to the level of the former substance during the last ACTH period, the major proportion of the 11-desoxy ketosteroids during all periods was probably derived from a C-19 precursor.

The steroid patterns during Period 6, following the termination of ACTH administration, show that both subjects manifested a selective and significant decrease in 11-desoxysteroids. This occurred despite the "masking" effect of the pooled urine collection and at a time when the 11-oxygenated steroids were at normal levels. During this "rebound," moreover, the proportion of etiocholanolone to androsterone did not undergo notable change. These findings may be interpreted as a further reflection of the existence of a selective pathway for androgen synthesis, as defined by the metabolites androsterone and etiocholanolone.

SUMMARY

The qualitative and quantitative aspects of urinary ketosteroid excretion have been studied in normal women of various ages. The data establish that the sum total of processes concerned with the biosynthesis, secretion and metabolism of the 11-oxygenated steroids examined is independent of chronological age in normal women. The secretion of 11-desoxy hormonal precursors, however, seems to diminish with increasing age. The chemical nature of the 11-desoxy steroid metabolites makes it likely that this alteration in adrenal function reflects the selective diminution in secretion of an "adrenal androgen." The response to stimulation with adrenocorticotrophic hormone has been studied in two normal women as well and was found comparable with that previously noted in normal males. The individual urinary ketosteroids were essentially at a constant level during successive periods of the menstrual cycle in two normal women.

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THE INCIDENCE OF INFECTION AMONG CONTACTS OF POLIOMYELITIS CASES[1]

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One of the epidemiological features which puzzled early students of poliomyelitis was the apparently erratic spread of the disease with rarely more than one case in a family. Subsequently, it became apparent that unlike measles among the common virus diseases, the majority of infections with poliomyelitis virus were either mild or inapparent and, therefore, went undetected. A number of epidemiological survey studies carried out during the past two decades indicated that actually a high infection rate was common among family contacts of poliomyelitis cases even though only one individual in the family became sick. Until recently, however, the laboratory methods available for studying the problem were largely limited to the isolation of virus by monkey inoculation, and the exact rates or degree of spread of this infection through families remained unknown. With the introduction of tissue culture methods into poliomyelitis research, it has been possible to carry out more extensive virological studies, and to relate virus isolation—or its absence—to the immune or antibody status of the individuals tested. The present study is concerned with poliomyelitis infection rates among close contacts of cases and indicates actually how high they may be. Tissue culture methods, which have been brought to bear on the problem, have been responsible for these results.

MATERIALS AND METHODS

Contacts of cases occurring in an epidemic in rural Ohio involving Wayne and Medina counties during the summer of 1952 formed the subjects for this study. The outbreak was a sharp one beginning in June and reaching a peak by the end of July. Overall attack rates in the two counties were 193 and 280 per 100,000, respectively, with mortality rates of 16 and 8.2 per 100,000 (1). Many of the cases occurred in small towns with a population of several hundred to a thousand. A relatively large number of families with multiple cases was noted.

Collection and preservation of specimens.[2] Our study was carried out between 20 June and 11 July, 1952. As soon as possible after a case of poliomyelitis arising in the study area was hospitalized or reported to the health officer, a visit was made to the family and neighborhood contacts by one of us. Inquiries were made as to whether individuals in these groups had had any illness within the previous few days or weeks; and whether any of the children had symptoms at the time of the visit. If the latter were found, the children were examined and their temperatures recorded.

A number of children who had not been ill enough to have been seen by a physician, nevertheless gave histories of recent short mild illnesses, with sore throat, headache, fever, and gastrointestinal symptoms, in various combinations, without evidence of central nervous system disease. These were considered by us to be possible or probable examples of *abortive poliomyelitis.* Among the rest, a few had vague symptoms which were not well enough defined to be classified as abortive poliomyelitis, and many were completely asymptomatic.

Contacts were classified either as: *household,* if they were part of the same family unit as the index case; or *daily,* if they had regular daily contact with the case, such as playmates, close neighbors, *etc.* The study was focussed on 91 contacts of index cases occurring in 28 families. Of the contacts studied, 54 were regarded as household, and 37 as daily. An effort was made to obtain throat swabs, rectal swabs, and blood specimens from as many of these individuals as possible, particularly those under 15 years of age. The specimens, collected from 91 persons, are listed in Table I by age groups. In addition, stool specimens, collected in the acute phase of the disease, were obtained from nine hospitalized cases, four of whom were index cases of the contacts studied. Throat and rectal swabs were placed in nitrocellulose tubes containing 1 ml. of 50 per cent

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TABLE I

Types of specimens collected from 91 contacts of poliomyelitis cases

Age	Numbers and types of specimens		
	T, R, Bl*	T, Bl	R, Bl
0–4	25		1
5–9	16	4	1
10–14	16	10	
15–19	3	10	
20+		5	
Total	60	29	2

* T = throat swab; R = rectal swab; Bl = blood.

glycerine and frozen on dry ice soon after collection. Stool specimens were frozen directly. Whole blood was collected in 20 ml. amounts in vacuum tubes[3] containing potassium oxalate as anticoagulant. The tubes of blood were centrifuged at 3000 rpm before freezing, thus layering the plasma, white cells and red cells. All specimens were transported to New Haven in the frozen state, and kept frozen at −20° until tested.

Throat and rectal swabs were prepared for inoculation by removing the cotton tips and squeezing them out thoroughly in 1 ml. of sterile saline. The eluate was spun at 3,000 rpm for 5 min. before being inoculated in 0.1 ml. amounts into tissue culture tubes. At first, monkey testicular roller tubes prepared by the plasma clot method (2) were used, 2 per specimen; all tubes showing any cellular degeneration were passed, while those in which no cytopathogenic effect was observed were discarded after 12 days' observation. Later, when it became apparent that monkey kidney tissue is more sensitive to small amounts of virus, all specimens which had been negative in testicular tubes were retested, using 4 to 6 kidney tubes per specimen, and following the same procedures for harvesting and passing as previously employed (3). By this method a number of positive results were obtained with specimens which had been negative in testicular tubes. Finally, the remaining negatives were retested by the suspended cell technique (4), using monkey kidney tissue and harvesting and passing to kidney epithelial outgrowth tubes at 5, 10, and 15 days after inoculation. No additional virus isolations resulted from this procedure.

Stools. Specimens were prepared for inoculation as described by Riordan, Ledinko, and Melnick (5). Twenty per cent suspensions were made with sterile distilled water, and spun at 3,000 rpm for 10 minutes. The supernatant fluid was then spun at 18,000 rpm for one-half hour in a refrigerated centrifuge. Following this, 3 ml. of supernatant was mixed thoroughly with ether and held at 4° C. overnight. After a further low speed centrifugation and removal of ether, 0.1 ml. was used as inoculum for each tissue culture tube. Monkey testicular tissue roller tubes were employed; they were followed in the same manner as those inoculated with throat and rectal swabs.

[3] Bekton-Dickinson "Vacutainer" tubes.

Typing of strains isolated. Virus strains isolated from throat swabs, rectal swabs, and stools, were typed according to methods previously reported from this laboratory (5). Viruses were tested against a 1:5 dilution of hyperimmune monkey sera, prepared by immunization with Brunhilde (Type I), Y-SK (Type II), and Leon (Type III) strains. Complete inhibition of virus multiplication by one type only was taken to mean specific neutralization.

The blood specimens were all tested for the presence of poliomyelitis virus as described elsewhere (6).

Antibody tests. The presence of neutralizing antibodies against the three types of poliomyelitis virus in the blood specimens was determined in tissue culture neutralization tests (2), using monkey kidney tissue tubes (3). A single determination was made with plasma in a 1:5 dilution against 100 tissue culture doses of poliomyelitis virus. Strains used in the neutralization tests were tissue culture adapted Brunhilde for Type I; Y-SK for Type II; and Leon for Type III.

RESULTS

Isolation of virus from contacts and cases

Poliomyelitis virus was isolated from various sources from 28 of the 91 individuals designated as contacts, and from all nine of the hospitalized paralytic and nonparalytic cases tested. All the strains of virus were found to belong to Type I. This suggests that in Wayne and Medina Counties, Ohio, the 1952 epidemic was due predominantly to a Type I strain.

Isolation of poliomyelitis virus from blood specimens

Virus was demonstrated in the blood of two contacts of paralytic cases, one aged 10 months,

TABLE II

Isolation of Type I poliomyelitis virus from throat and/or rectal swabs from 91 contacts of poliomyelitis cases

Specimens	Contacts	Type I virus isolation by specimens	
		Results	Number contacts
Throat and rectal swabs	60	T*+, R*+	8
		T+, R−	9
		T−, R+	7
		T−, R−	36
Throat swabs only	29	T+	4
		T−	25
Rectal swabs only	2	R+	0
		R−	2

* T+ and R+ or T− and R− indicate throat and rectal swabs positive or negative for Type I poliomyelitis virus, respectively.

who 3 days later developed non-paralytic poliomyelitis; and one aged 17 years who remained asymptomatic. In addition viremia was detected in four children in one family who had no history of exposure to a known case. Three of these children had the "minor illness syndrome," and one was asymptomatic. A detailed report of these findings has appeared elsewhere (6).

Isolation of poliomyelitis virus from throat and rectal swabs

The results of the tests on the 91 contacts are shown in Table II in terms of the distribution of positive specimens, *i.e.*, whether throat swabs or rectal swabs or both. The relatively large number with a positive throat swab and a negative rectal swab is perhaps a reflection of the method used; although rectal swabs are convenient and practical for survey purposes, they are less apt to yield virus than are stool specimens. However, with a rectal swab at least one knows from which child each specimen comes, and there is little chance for cross contamination within individual households.

The relationship of age and type of specimens tested to the number which were positive for virus is considered in Table III. No significant differences were noted in the age groups up to 15 years as to the frequency of virus isolation. There were more positive results when both throat and rectal swabs were tested, rather than either alone.

The type of exposure to a known case experienced by each individual, whether *household* or *daily*, was related to the number of persons who became infected; this is illustrated in Table IV,

TABLE III

Relation of age to the isolation of Type I poliomyelitis virus from 28 of 91 contacts of cases

Age	Specimens tested		
	T+R*	T	R
0–4	13/25†		0/1
5–9	6/16	0/4	0/1
10–14	5/16	2/10	
15–19	0/3	2/9	
20+		0/6	
Totals	24/60	4/29	0/2

* T and R = throat and rectal swabs.
† Numerator = number positive for virus; denominator = number tested.

TABLE IV

Relation of type of exposure to the readiness with which Type I poliomyelitis virus was isolated from 24 of 60 contacts whose throat and rectal swabs were both tested

Age	Type of contact	
	Household	Daily
0–4	11/13*	2/12
5–9	4/8	2/8
10–14	5/12	0/4
15–19	0/3	
Totals	20/36(56%)	4/24(17%)

* Numerator = number positive; denominator = number tested.

in which are included only the 60 contacts from whom both throat and rectal swabs were available for testing. Of the *household* contacts, 20 of 36 were found to be infected, while for *daily* contacts the figures were 4 of 24. The group which yielded the largest number of positive results was that composed of 0 to 4-year-old household contacts, in which 11 of 13 (in 10 households) were positive in contrast to 2 of 12 for the daily contacts in this age group. When data on all 91 contacts of cases were tabulated, the results turned out similarly: 24 of 54 (44 per cent) household contacts were positive for virus, while 4 of 37 (11 per cent) of daily contacts were positive. When analyzed on an age basis it was found that 22/42 (52 per cent) of those under 15 years were excreting virus in the household group, in contrast to 2/12 (17 per cent) of those 15 years and over; 4 of 31 (13 per cent) daily contacts under 15 were positive, but none over 15 yielded virus.

Antibody studies

In order to evaluate the trends shown in these virus isolations from individuals of different ages and with different histories of contact, information as to the antibody status of the group under study was sought, for it seemed likely that in certain groups no virus was isolated because the individuals possessed antibodies and were, therefore, presumably immune. This question was analyzed by determining the poliomyelitis neutralizing antibody pattern for Types I, II, and III in those excreting virus and in those from whom no virus was isolated. Of the 28 individuals positive for Type I virus, 19 (68 per cent) had specific antibodies, while 9 were early enough in the course

TABLE V

Type I neutralizing antibody pattern in 36 of 60 contacts whose throat and rectal swabs were negative for Type I poliomyelitis virus

Age	Type of contact	
	Household	Daily
0–4	2/2*	8/10
5–9	4/4	6/6
10–14	7/7	3/4
15–19	1/3	
Totals	14/16(87%)	17/20(85%)

* Numerator = no. positive for antibodies; denominator = no. tested.

of infection to be still without a demonstrable Type I antibody response. Table V shows the pattern for Type I antibodies found in the 36 whose throat and rectal swabs had been tested and found negative for virus. The results indicate that a high percentage of both household and daily contacts, 87 per cent and 85 per cent, respectively, had Type I antibodies, either as a result of recent or remote infection.

A similar pattern was obtained when the entire group was analyzed as shown in Table VI, which summarizes the results of virus isolations from 28 of the total 91 contacts, and the antibody status of the 63 negative for virus. Of 42 household contacts under 15 years of age, 22 (52 per cent) were excreting virus, and all 20 of those negative for virus had Type I antibodies. For the age group 15 years and over, virus isolations were fewer, and only 58 per cent of those negative for virus had antibodies. The difference between household and daily contact groups as to virus isolation was great, but of the daily contacts, the per cent *either* excreting virus *or* possessing antibodies is similar to that for the household group: 90 per cent of those under 15 years, and 50 per cent of those 15 years and over. Again, whether or not the neutralizing antibodies present were the result of recent infection, *i.e.*, in the current epidemic—or whether they represented infection in some previous year is not known; but an analysis of the antibody pattern to Types I, II, and III poliomyelitis virus suggests that in all probability a number of infections were acquired during the current epidemic (Table VII). Thus, a significant number of contacts in the age groups 0 to 4 and 5 to 9 had only Type I antibodies, or Type I plus II and/or III. The possibility exists that some of the Type II and III antibodies represent heterotypic responses to recent Type I infections (7). That the Type I antibodies could be heterotypic is unlikely, since no other virus types were isolated in any of the families or hospitalized cases.

Relation of symptoms to the isolation of virus

With the exception of three children in the contact group who subsequently developed nonparalytic poliomyelitis (and four others who were hospitalized secondary cases), illnesses in various individuals in the group were characterized by non-

TABLE VI

Summary of Type I poliomyelitis virus isolations from contacts, and Type I antibody status of those negative for virus

Age		Type of contact	
		Household	Daily
0–14	Number	42	31
	Virus positive	22	4
	Virus negative	20	27
	Virus neg., antibody pos.	20	24
	Summary:		
	Virus pos./No. tested	22/42(52%)	4/31(13%)
	Virus *or* antibody pos./No. tested	42/42(100%)	28/31(90%)
15+	Number	12	6
	Virus positive	2	0
	Virus negative	10	6
	Virus neg., antibody pos.	5	3
	Summary:		
	Virus pos./No. tested	2/12(17%)	0/6
	Virus *or* antibody pos./No. tested	7/12(58%)	3/6(50%)
Total	Virus pos./No. tested	24/54(44%)	4/37(11%)
	Virus *or* antibody pos./No. tested	49/54(91%)	31/37(84%)

TABLE VII

Type I, II, and III poliomyelitis antibody patterns of 63 contacts negative for poliomyelitis virus

Contact	Age	Total number	Type I only (homotypic)*	Antibodies against Type I+II+/or III (homo- and heterotypic)	Type II+ or III only (heterotypic)	No antibodies to any type
Household	0–4	2	2			
	5–9	6	1	5		
	10–14	12		12		
	15+	10		4	5	1
Daily	0–4	11	6	3		2
	5–9	9	5	3	1	
	10–14	7		4	3	
	15+	6	1	2	1	2

* "Homotypic" refers to the Type I epidemic strain.

specific symptoms usually considered representative of the *minor illness* or abortive form of poliomyelitis which cannot be diagnosed clinically. These were slight fever, anorexia, sore throat, and headache, usually lasting not more than 24 to 48 hours; a few had more vague symptoms, such as a "cold," constipation, irritability, *etc.* The analysis of the relationship between the presence or absence of symptoms and the isolation of virus (Table VIII) showed that among the 91 household or daily contacts, 14 of 26 (54 per cent) of individuals *with symptoms* either during the three weeks prior to the collection of specimens, or at the time specimens were collected, were positive for virus; 11 of these had symptoms typical of the minor illness, and three had "colds." Of those asymptomatic during the same periods, 14 of 65 (23 per cent) yielded Type I poliomyelitis virus; presumably then, these 14 individuals were examples of inapparent infections. The percentage of virus isolation was higher in those with symptoms of the minor illness, especially in the younger children. The question as to how many failures to isolate virus were due to the fact that the individuals possessed antibodies and could, therefore, be considered immune, was investigated. When such antibody positive ("immune") individuals who were virus negative were eliminated from the analysis, then the score 14 of 17 (82 per cent) of those with symptoms were positive for virus, while 14 of 26 (54 per cent) of those who remained asymptomatic were infected, as demonstrated by the isolation of virus. The difference is not statistically significant. The difference between the symptomatic and asymptomatic group as to ease of virus isolation also disappears if all those negative for virus but positive for antibodies are assumed to be examples of inapparent infection during the current epidemic.

Clinical manifestations—Ratio of inapparent to apparent infection among contacts

The data were next analyzed from the point of view of clinical manifestations or their absence. In this analysis, the index cases were eliminated and only their household contacts (plus four additional family contacts who were secondary hospitalized cases) were included; the stool of two of these latter had been tested and found positive for Type I poliomyelitis virus.

Table IX indicates the various responses of these 58 individuals. The incidence of infection was high indeed, for of 31 "susceptibles," 28 be-

TABLE VIII

The presence of symptoms as compared with the isolation of Type I poliomyelitis virus from 91 close contacts

	Symptoms present		Symptoms absent	
	Type of contact		Type of contact	
Age	Household	Daily	Household	Daily
0–4	6/6*	2/4	5/7	0/9
5–9	3/3	1/2	1/7	1/9
10–14	1/4	0/2	6/15	0/5
15+	1/3	0/2	1/9	0/4
Totals	11/16	3/10	13/38	1/27
	14/26(54%)		14/65(23%)	
Total positive if "immune" (*i.e.*, antibody pos.) eliminated from virus neg. group	14/17(82%)		14/26(54%)	

* Numerator = number of individuals positive for Type I virus; denominator = number tested.

TABLE IX

Clinical responses of familial associates of poliomyelitis cases

Age	Total number	"Susceptibles"*		Clinical manifestations 28 infected contacts			
				Number with:			
		Number	Per cent infected	Inapparent infection	Abortive	Non-paralytic	Paralytic
0–4	13	11	100	5	3	3	0
5–9	14	8	100	1	3	2	2
10–14	19	7	100	6	1	0	0
15+	12	7	29	2	0	0	0
Totals	58	31	90	21		7	

* "Susceptibles" as used here include 1) all contacts from whom virus was isolated; 2) two frank cases not tested for virus; 3) contacts negative for virus and negative for Type I antibodies.

came infected. All of the 7 frank cases were in children under 10 years of age, but the numbers are too few to draw conclusions about the clinical form of disease in relation to age. However, it is possible to compare the ratio of inapparent and abortive infections to frank clinical cases. Of the 28 infected persons, 21 had either inapparent or abortive poliomyelitis, while 7 had the frank clinical disease, either paralytic or nonparalytic, a ratio of 3:1.

DISCUSSION

The incidence of infection among contacts of poliomyelitis cases has been studied by many investigators, and there is ample evidence that both asymptomatic infections and minor illnesses are common in familial and other associates of cases. Only recently, however, since the introduction of tissue culture methods, has it been feasible in large studies to correlate the isolation of virus with the presence or absence of antibodies to the infecting type of poliomyelitis virus. In results recently published by Brown, Rabson, and Schieble (8) in which the excretion of virus and development of antibodies was studied in 55 family contacts who had received gamma globulin, the results were remarkably similar to those of the present investigation. The above authors, conducting their study in Michigan in the summer of 1953, found that 23 of the 55 household contacts were excreting virus; that 27 (85 per cent) of those negative for virus already had antibodies to the family strain. In our study conducted in Ohio during the summer of 1952, 24 of 54 familial associates were found to be positive for the family type of virus, and 25 (83 per cent) of those from whom no virus was isolated already had antibodies. Thus in both studies, 91 per cent of family contacts were either excreting virus, *or* possessed antibodies to the family type. Similar high infection rates among familial associates have been reported earlier by Brown and Ainslie (9) and, more recently, by Bodian and Paffenbarger (10).

In considering the ease of virus isolation from symptomatic as compared to asymptomatic contacts, as might be expected, a higher percentage of positives was obtained in those with symptoms (54 per cent) in contrast to the asymptomatic group (23 per cent). The difference is probably explained by the fact that in the asymptomatic group, many possessed Type I antibodies and were therefore "immune" because of previous exposure, and others, who had been infected earlier in the current epidemic, were actually examples of inapparent infection.

As to the clinical responses of family contacts of cases, it is apparent from Table IX that the 3:1 ratio of inapparent to apparent infections is considerably different from the 100:1 or greater ratio usually given for the general population. Even if the 25 family contacts negative for virus but possessing Type I antibodies (Table VI) are all considered to be examples of inapparent infection during the current epidemic, the ratio is still only 46:7, or approximately 7:1. The results suggest that infected families in which a frank case has occurred form a special, highly exposed group, with a relatively high risk of clinical disease. A number of factors including virus dosage may be responsible for this situation. On the experimental side, for instance, it has been observed

by Sabin and Winsser (11) that small doses of virus repeatedly fed to cynomolgus monkeys produce predominantly inapparent infection; in contrast, animals fed large doses of virus are prone to develop paralytic poliomyelitis in a high per cent of instances. By analogy, family associates of a case might be considered to be a special group, probably exposed to large amounts of virus, and hence having a relatively high attack rate of paralytic and nonparalytic infections. In contrast, in a normal population which lives through an epidemic without known exposure to a case, a high incidence of inapparent infection is demonstrable. Such a group was described by Melnick and Ledinko (12) who measured the conversion rate from negative to positive antibody status in 250 normal children who lived in Winston-Salem, N. C., during an epidemic there in 1948. The ratio of inapparent infections in the group studied to reported cases in the community was calculated to range from 175:1 in infants under 1 year to 62:1 and 95:1 for children 5 to 9 and 10 to 14 years of age, respectively. The high inapparent infection rate in such individuals with no known exposure might be correlated with the size of the infecting dose: as with monkeys fed small doses of virus, minimal exposure produced inapparent infection and immunity. Thus it has been said (and there is increasing evidence on this point [13]) that to some extent paralytic poliomyelitis "breeds" paralytic poliomyelitis, while asymptomatic infections in the population at large breed asymptomatic infections.

In any event, the results of the present study are in line with others (8–10) in pointing to an extraordinarily high infection rate among susceptible individuals closely exposed to a case. It may be assumed on the basis of the evidence presented that by the time a case of poliomyelitis occurs in a family, virtually all other members of the family who are not immune by virtue of previous exposure and infection have become infected, whether or not they develop symptoms. Thus, poliomyelitis may be said to rank with measles as to degree of contagiousness in a family setting.

SUMMARY

1. The incidence of infection among 91 contacts of poliomyelitis cases has been studied during an epidemic due to Type I. Virus isolations as a measure of infection have been correlated with immune status, *i.e.*, the presence or absence of antibodies.

2. The incidence of infection among household and daily contacts of diagnosed cases was high. If only antibody negative (and therefore presumably susceptible) contacts are considered, the infection rates for those under 15 years of age were 100 per cent for household contacts, and 87 per cent for daily contacts.

3. Type I poliomyelitis virus was isolated with greater frequency from those with symptoms than from those who remained asymptomatic. However, many of the asymptomatic group were demonstrated already to possess antibodies to the family type of virus; when these were eliminated, there was no significant difference in the ease of virus isolation from individuals with symptoms as compared to those who remained asymptomatic.

4. In analyzing the clinical response to poliomyelitis virus infection among family associates of cases, it was found that the ratio of inapparent to apparent infection was approximately 3:1 (and not higher than 7:1). It is suggested that the high incidence of apparent infections in the group studied (compared to the ratio for the general population) might be related, in part at least, to factors of exposure and dosage: in a family in which a frank case occurs, exposure is of a high degree, the dosage of virus is presumably large, and the incidence of paralytic and nonparalytic infections is correspondingly high.

5. On the basis of virus isolations and serological evidence, poliomyelitis infection may be said to be as contagious as measles among susceptible individuals in a family setting.

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BIOCOLLOIDS OF URINE IN HEALTH AND IN CALCULOUS DISEASE. II. ELECTROPHORETIC AND BIOCHEMICAL STUDIES OF A MUCOPROTEIN INSOLUBLE IN MOLAR SODIUM CHLORIDE [1]

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A method for recovering the total biocolloids of urine by a combination of dialysis and ultrafiltration has been described (1). These biocolloids have been separated into two groups on the basis of their solubility in molar sodium chloride. The electrophoretic properties of those components which are soluble in molar sodium chloride have been reported, both for normal subjects and for patients with developing renal calculi (2). The urinary biocolloids which are insoluble in molar sodium chloride form the subject for the present study.

A virus inhibitory urinary mucoprotein which is insoluble in sodium chloride solutions of 0.58 M, and only partially soluble in sodium chloride solutions as low as 0.14 M was first discovered by Tamm and Horsfall in 1950 (3). In 1952, further reports by Tamm and Horsfall (4), Gottschalk (5), and Odin (6) indicated that this mucoprotein was a potent inhibitor of hemagglutination by influenza viruses. The physical homogeneity, molecular weight, and axial ratio of this substance have been delineated by Tamm, Perlmann, Horsfall, Bugher, and Porter, employing electrophoretic (7), ultracentrifugal (8), and electronmicroscopic (9) methods. Burnet (10) has reported alterations in the capacity of this mucoprotein to inhibit viral hemagglutination following periodate treatment, and loss of its ability to produce agglutination of erythrocytes following treatment with 5 M urea solution. Curtain (11) found that the solubility of this mucoprotein in solutions of sodium chloride was much increased by treatment with 6.5 M urea solution. He observed that the presence of calcium, ionic strength $\mu = 0.023$, and magnesium, ionic strength $\mu = 0.017$, prevented urea from altering the viscosity and the hemagglutinating and virus-inhibitory properties of the mucoprotein (12).

The current studies have demonstrated that ultrafiltration of dialyzed urine, followed by extraction of the retained gel with molar sodium chloride, yields a residue which in chemical composition is indistinguishable from the urinary mucoprotein of Tamm and Horsfall. In the present report the amount of NaCl-insoluble mucoprotein in urine of normal subjects is compared with that in urine from patients with calcigerous renal calculi. The effect of solutions with a pH of 12.3 on the solubility of this mucoprotein, and the electrophoretic behavior of the mucoprotein after exposure to such solutions are also reported.

MATERIAL AND METHODS

Studies were performed on five to ten 24-hour specimens of urine from each of 8 normal subjects, 14 patients with calcigerous renal calculi, and 2 patients who had bilateral cutaneous ureterostomies as a result of cystectomy performed for carcinoma of the bladder. In an effort to reduce bacterial activity to a minimum, all patients received intensive therapy with antibiotic or chemotherapeutic agents, or both, before and during the period of study.

All the buffer solutions used were of 0.1 ionic strength, and were prepared according to the method described by Miller and Golder (13). Determinations of pH were made with the glass electrode. Conductivity studies were made in a Shedlovsky type cell at 0.5 C. Electrophoretic studies were carried out in the Aminco apparatus, in the manner previously described (2). Analyses for nitrogen, carbon, hydrogen, sulfur, and sulfate were done by a commercial analyst (14). The hexose, hexosamine, fucose, and "sialic acid" determinations were made by Dr. Richard Winzler (15), using techniques which he has described (16). The analyses for nitrogen and for hexose, hexosamine, and "sialic acid" were also done in our own laboratory. Purified samples of orosomucoid (human serum mucoprotein, MP-1) were provided by

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Dr. Winzler as a standard for the "sialic acid" determinations. It was noted that the color of the urinary mucoprotein preparations developed with the diphenylamine reaction is not identical to the color of those developed in the standards prepared from serum mucoprotein; hence the values for "sialic acid" in urinary mucoprotein may not be entirely comparable to values obtained by similar determinations performed on serum mucoproteins.

Twenty-four-hour specimens of urine were collected into sterile, refrigerated containers and were kept at temperatures of 1 to 5 C. during the entire study. Specimens with a specific gravity of more than 1.010 were diluted with distilled water, since with moderate centrifugal force the material under study sediments from concentrated urine (4). After adjustment of the specific gravity, all specimens were centrifuged to remove formed elements. The total biocolloids of each specimen were then recovered by cold dialysis and ultrafiltration, and soluble components were removed by extraction with M sodium chloride in the manner previously described (2).

The dry weights of the insoluble residues contained in one to five 24-hour urine specimens from each subject were determined after dialysis in the cold against distilled water, lyophilization, and subsequent drying *in vacuo* for 24 hours at 20 C. The lyophilized residues were then used for elementary and carbohydrate analyses.

The remaining insoluble residues which had been frozen for storage were prepared for electrophoresis in the following manner: The residue was suspended in glycine buffer of pH 12.3, and dialyzed against the same buffer for 24 hours at 1 C., with frequent gentle agitation. Any insoluble residue was removed by centrifugation, and portions of the clear supernatant fluid were equilibrated by dialysis with buffers of pH 4.5, 8.6, and 12.0. The times and potential gradients used were as follows:

At pH 4.5—10,800 sec., 6 volts per cm.
At pH 8.6—7,200 sec., 7.4 volts per cm.
At pH 12.0—7,200 sec., 6 volts per cm.

All studies were made at 0.5 C. and in accordance with techniques previously described (2). After electrophoresis, the degraded mucoprotein was recovered by dialysis against distilled water, followed by lyophilization. This residue was submitted to elementary and carbohydrate analyses.

Both normal serum proteins and purified serum mucoprotein (MP-1), to which Winzler has recently given the name *orosomucoid* (16), were used to obtain comparative electrophoretic patterns.

RESULTS

Dry weights of the residues insoluble in molar sodium chloride

The weights of the residues from normal individuals were strikingly constant, ranging from 38 to 56 mg. in 18 determinations performed on 24-hour urine specimens. The average yield from 3 normal subjects studied for five consecutive days was 44 mg. per day. The 24-hour residues from the urine of patients with calculous disease demonstrated a marked quantitative variation, not only between individuals but from day to day for the same individual. The dry weights of these residues ranged from 147 to 1,040 mg. per 24 hours. For the two patients who had cutaneous ureterostomies, the dry weights of the residues insoluble in molar sodium chloride were, respectively, 18 and 12 mg. per 24 hours.

Chemical composition of the residues insoluble in molar sodium chloride

The results of chemical analyses of the NaCl-insoluble mucoprotein recovered in our laboratory and other laboratories are given in Tables I and II. All analyses were performed on material which was first dialyzed against distilled water and recovered by lyophilization, then dried *in vacuo* at room temperature to a constant weight. These results indicate the similarity between the virus inhibitory mucoprotein of Tamm and Horsfall and the NaCl-insoluble portion of the residue recovered by ultrafiltration of urine.

We have been unable to demonstrate any significant qualitative difference in this material between the normal subjects and 11 of the 14 patients with calculous disease. In 3 patients who had renal calculi and persistent urinary infection with *B. proteus* the hexosamine content of the mucoprotein was decreased (Table II). No other variations in carbohydrate content were noted in the mucoprotein recovered from these patients. These were the only patients from whom this organism was cultured in the present study.

Electrophoretic studies of the residues insoluble in molar sodium chloride

In phosphate buffers of pH 6.8 and ionic strength 0.05, the lyophilized material was found to have a mobility of -8.6×10^{-5} cm.^{2} per volt sec. This figure agrees with that reported by Perlmann, Tamm, and Horsfall (7), who also used the phosphate-soluble portion of lyophilized mucoprotein.

TABLE I

Inorganic analysis of urinary mucoprotein as reported by different investigators (expressed as per cent of dry weight)

Author	Nitrogen	Carbon	Hydrogen	Sulfur			Phosphorus	Acetyl groups
				Sulfate	Other	Total		
Tamm and Horsfall (4)								
Preparation 1	12.2	48.8	6.65			3.09 (Parr Bomb)	None	
Preparation 2	11.8	46.4	6.84			1.82 (Carius)	None	
Odin (6)								
Preparation 1	11.2							
Preparation 2	10.9					0.4	0.09	
Preparation 3	10.7					0.8	0.16	
Present study						(Carius)		
Normal Subjects								
As recovered	12.2	48.9	6.9	1.43	0.37	1.80	None	0.27
After treatment at pH 12.3	10.3	49.2	6.9	1.19	0.40	1.59	None	1.35
Calculous disease								
(Av. for 11 patients*)								
As recovered	12.9	49.4	7.0	1.21	0.38	1.59	None	0.28
After treatment at pH 12.3	11.3	50.9	7.0	1.26	0.38	1.64	None	1.20

* Not including 3 patients with persistent urinary infection with *B. proteus*.

When the NaCl-insoluble residue was suspended in veronal buffer of pH 8.6 immediately after recovery from the ultrafilter and prior to freezing or other treatment, it was found to migrate toward the anode with a mobility of -8.3×10^{-5} cm.^{2} per volt sec. Whether this unfrozen material can be considered "soluble" in this buffer is debatable, since it can be sedimented by centrifugation at $500 \times g$ for 60 minutes.

Figure 1 illustrates by electrophoretic patterns the presence of this mucoprotein in the entire protein residue recovered by ultrafiltration of uri-

TABLE II

Carbohydrate content of urinary mucoprotein as reported by different investigators (expressed as per cent of dry weight)

Author	Hexose			Hexosamine	Fucose	"Sialic acid"		Total
	Galactose	Mannose	Total			Ehrlich	Bial	
Gottschalk (5)	5.4	2.7	8.1	7.6	1.0			
Odin (6)								
Preparation 1			6.8	6.4	0.7	8.2	7.3	22.1
Preparation 2			8.4	9.4	1.1	9.2	9.1	28.1
Preparation 3			9.0	7.0	1.1	9.4	9.1	26.5
Average			8.3	7.7	1.0	8.9	8.5	25.9
Present study								
Normal subjects								
As recovered			8.4	7.1	1.1	9.1		25.7
After treatment at pH 12.3			8.3	2.3	1.2	8.8		20.6
Calculous disease								
(Av. for 11 patients*)								
As recovered			8.1	7.4	1.0	9.0		25.5
After treatment at pH 12.3			8.0	2.5	0.8	8.8		20.1

* Not including 3 patients with *B. proteus* infection whose mucoproteins gave hexosamine contents of 2.5, 2.5, and 4.8 per cent without significant variation in other carbohydrate components.

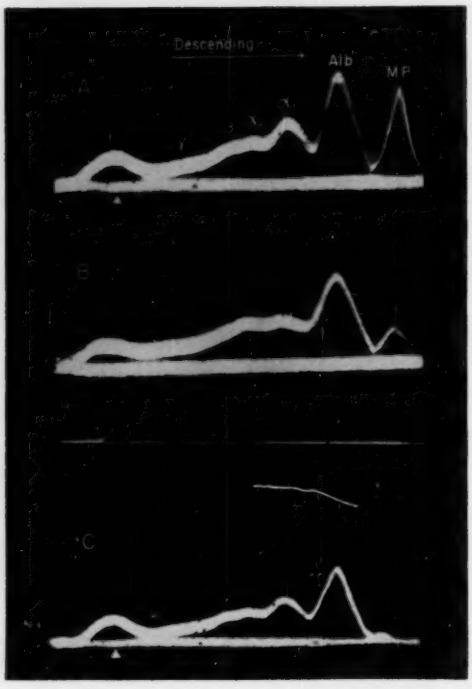

FIG. 1. ELECTROPHORETIC MIGRATION PATTERNS OF URINARY PROTEINS FROM A PATIENT WITH PYELONEPHRITIS (B. COLI)

Slit diaphragm, cylindrical lens patterns of boundaries descending toward the anode. Veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 6.6 volts per cm., time, 7,200 sec.

A. Entire gel recovered from ultrafiltration of urine was suspended in buffer without freezing or centrifugation and electrophoresis performed after equilibration dialysis against buffer. MP—Tamm and Horsfall mucoprotein, Alb.—Albumin, ‹—salt boundary. The MP boundary has been retouched to facilitate copying.

B. Entire gel recovered from ultrafiltration of urine treated as A with the exception of centrifugation at $500 \times g$ for 1 hr. just prior to electrophoresis. The sediment was identified as T & H mucoprotein by electrophoresis after treatment at pH 12.3.

C. Entire gel recovered from ultrafiltration of urine treated as A with the exception that NaCl was added to make the whole 0.58 M with respect to NaCl, centrifuged at $500 \times g$ for 1 hr., and electrophoresis performed after equilibration dialysis against buffer. Sediment identified as T & H mucoprotein by nitrogen and carbohydrate content.

nary proteins from a patient with pyelonephritis. The entire gel recovered from the ultrafilters was suspended in veronal buffer of pH 8.6 immediately after recovery, and this solution was then divided into three equal parts. Portion "A" was subjected to electrophoresis without further treatment other than dialysis against the buffer for 24 hours (Figure 1-A). A large component was observed to migrate with a mobility of -7.44×10^{-5} cm.^{2} per volt sec. This figure is 25 per cent greater than the mobility of the albumin fraction, and is exactly the same as the mobility of the fast component "fc-1" (2). Part "B" was dialyzed against the buffer for 24 hours and centrifuged at $500 \times g$ for 60 minutes prior to electrophoresis. The loss of this large component (MP in Figure 1) by sedimentation during centrifugation is illustrated in Figure 1-B. Sodium chloride was added to portion "C" to make a 0.58 M solution with respect to sodium chloride. This was then centrifuged for one hour at $500 \times g$, dialyzed against veronal buffer for 48 hours, and subjected to electrophoresis. The loss of the component with a mobility greater than albumin is illustrated in Figure 1-C.

Solubility of the residues insoluble in molar sodium chloride

Freezing of the residue remaining after extraction with molar sodium chloride was found to exert a profound effect on its solubility. When this material was recovered from a pool formed by freezing successive 24-hour samples of urinary biocolloids, the residue was highly insoluble in veronal buffer of pH 8.6 (2). However, it was possible to obtain suspensions of 1 to 2 per cent of the frozen mucoprotein in this buffer, and these appeared stable enough for an attempt at electrophoretic studies. At 0.5 C. these suspensions formed thixotropic gels within the electrophoresis cells, and the bulk of the material either precipitated spontaneously or remained immobile in the position of the "salt" boundaries. A barely detectable quantity was usually observed to migrate, with a mobility of approximately -8×10^{-5} cm.^{2} per volt sec. This gelation occurred whether the residues were obtained from normal urine or from patients with calculous disease, and whether the material was recovered by precipitation with 0.58 M sodium chloride or by ultrafiltration.

It was considered that the solubility of the mucoprotein might be affected by the presence of al-

kaline earth metals bound from the urine, either prior to precipitation with sodium chloride or by filtration of dialyzed urine through the gel as it formed on the collodion membrane. In an effort to remove all the alkaline earth metals, the NaCl-insoluble residue was suspended in distilled water and dialyzed for 72 hours against three changes of a solution prepared by dissolving 5 gm. of tetrasodium ethylenediaminetetra-acetate in 1 liter of acetate buffer of pH 4.5. The final pH of this solution was 7.4. Samples of both dialyzed and undialyzed material were filtered after being treated at 100 C. in 10 per cent nitric acid. The filtrate was concentrated and examined spectroscopically (17). Although no magnesium was detected in either sample, both contained calcium; there appeared to be less calcium, however, in the samples treated with ethylenediaminetetraacetate. After dialysis against EDTA the mucoprotein appeared to be more easily suspended in buffers of ionic strength 0.1, but centrifugation at $500 \times g$ for 60 minutes resulted in sedimentation of the suspended material.

Modification of the residues insoluble in molar sodium chloride by treatment at pH 12.3

When the NaCl-insoluble mucoprotein was suspended in buffers of pH 12.3 at 1 to 5 C., it became progressively converted to a highly soluble form. Within the first 24 hours approximately one-half became soluble, and after 72 hours only a small gum-like residue remained, amounting to approximately 6 per cent by weight of the original material. Treatment of the mucoprotein at pH 12.3 for ten days did not appear to result in further alteration of its properties. This highly soluble form appeared to be a degradation product of the original material, having lost two-thirds of the hexosamine content (Tables I and II). This soluble product was electrophoretically homogeneous and retained a net negative charge throughout the pH range of 4.5 to 12. It was possible to reproduce this material from the NaCl-insoluble residue of every subject in the present series, even when it had been previously lyophilized or frozen.

In Figure 2 the electrophoretic patterns of normal human serum are compared with those of the degraded urinary mucoprotein at pH 4.5. The

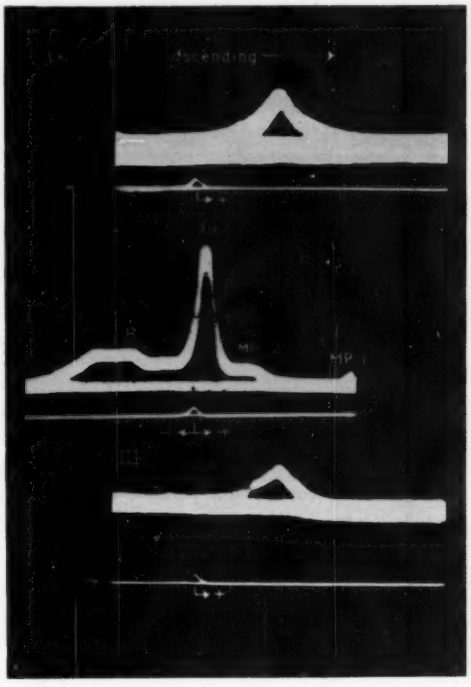

FIG. 2. COMPARATIVE ELECTROPHORETIC PATTERNS AT pH 4.5 OF URINARY MUCOPROTEIN TREATED AT pH 12.3 AND NORMAL HUMAN SERUM PROTEINS

I. TREATED URINARY MUCOPROTEIN FROM NORMAL SUBJECT

II. HUMAN SERUM PROTEINS FROM NORMAL SUBJECT

III. TREATED URINARY MUCOPROTEIN FROM PATIENT WITH RENAL CALCULI

Slit diaphragm, cylindrical lens patterns of boundaries ascending toward the anode. Acetate buffer, pH 4.5, ionic strength 0.1, potential gradient 6.0 volts per cm., time 10,800 sec.

degraded mucoprotein retains a net negative charge and migrates toward the anode with a mobility greater than that of the glycoprotein, MP-2, and much less than that of the orosomucoid, MP-1, of the serum.

Figure 3 is a series of Schlieren diagrams obtained from a mixture of degraded urinary mucoprotein and orosomucoid at pH 4.5. Under these conditions the urinary mucoprotein had a mobility of -2.09×10^{-5} cm.^{2} per volt sec., as compared with -3.96×10^{-5} cm.^{2} per volt sec. for this preparation of serum orosomucoid.

Figure 4 is a comparison of the electrophoretic

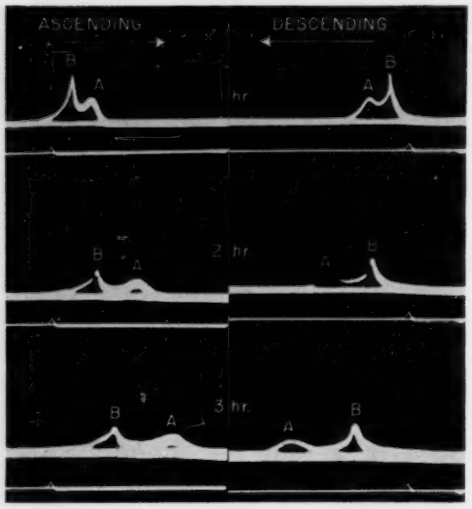

FIG. 3. COMPARATIVE ELECTROPHORETIC PATTERNS AT pH 4.5 OF URINARY MUCOPROTEIN TREATED AT pH 12.3 AND NORMAL HUMAN SERUM OROSOMUCOID (MP-1)

Acetate buffer, pH 4.5, ionic strength 0.1, potential gradient 6.2 volts per cm. A. Orosomucoid (serum mucoprotein MP-1). B. Treated urinary mucoprotein from normal subject.

patterns of the degraded urinary mucoprotein and normal human serum at pH 8.6. The urinary mucoprotein has a mobility of -5.56×10^{-5} cm.^{2} per volt sec., as compared with -5.94×10^{-5} cm.^{2} per volt sec. for albumin and -5.07×10^{-5} cm.^{2} per volt sec. for the alpha-1 globulin under similar conditions.

In Figure 5 the electrophoretic patterns of degraded urinary mucoprotein and normal serum are compared at pH 12.0. The urinary mucoprotein has a mobility less than that of any of the components of normal serum, which are poorly separated under these conditions.

DISCUSSION

Approximately half of the total biocolloids recovered from normal urine by ultrafiltration are insoluble in molar sodium chloride. The extraction of this residue in a variety of buffers ranging from pH 4.5 to 12.0 and with ionic strengths of 0.025, 0.05 and 0.1 have failed to produce evidence that this residue contains more than one mucoprotein. The results of the current studies indicate that this residue is the same material as the virus inhibitory mucoprotein of Tamm and Horsfall (4).

The previously reported techniques for recovery of virus inhibitory mucoprotein from the NaCl-insoluble precipitate of urine included a step in which an insoluble residue was discarded (4, 18). Hence it was thought that the mucoprotein of Tamm and Horsfall was more soluble than that which forms the subject of the present study, and we suggested that the NaCl-insoluble mucoprotein recovered from urine by ultrafiltration should be designated *uromucoid* (19). The present studies and other recent publications (8, 9), however, in-

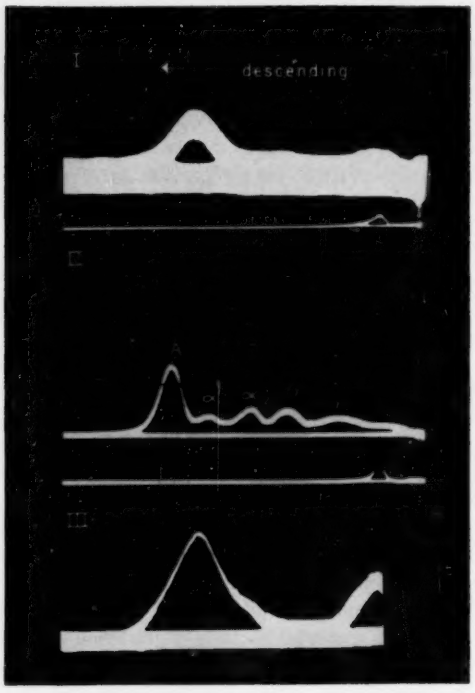

FIG. 4. COMPARATIVE ELECTROPHORETIC PATTERNS AT pH 8.6 OF URINARY MUCOPROTEINS TREATED AT pH 12.3 AND NORMAL HUMAN SERUM PROTEINS

I. TREATED URINARY MUCOPROTEIN FROM NORMAL SUBJECT

II. NORMAL HUMAN SERUM PROTEINS

III. TREATED URINARY MUCOPROTEIN FROM PATIENT WITH RENAL CALCULI

Slit diaphragm, cylindrical lens patterns of boundaries descending toward the anode. Veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 7.4 volts per cm., time 7,200 sec.

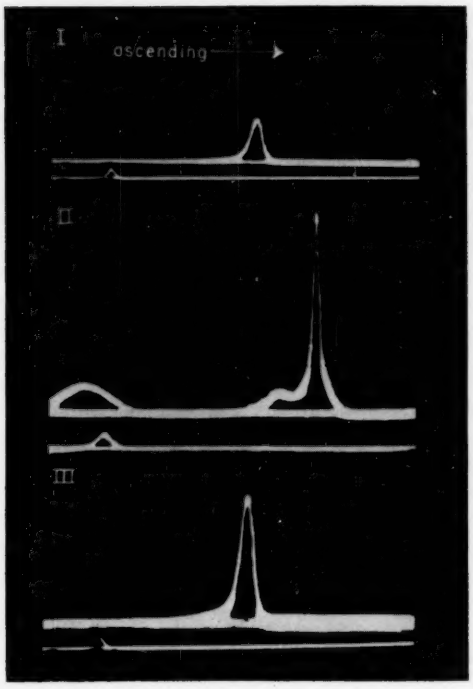

FIG. 5. COMPARATIVE ELECTROPHORETIC PATTERNS AT pH 12.0 OF URINARY MUCOPROTEINS TREATED AT pH 12.3 AND NORMAL HUMAN SERUM PROTEINS

I. TREATED URINARY MUCOPROTEIN FROM NORMAL SUBJECT

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III. TREATED URINARY MUCOPROTEIN FROM PATIENT WITH RENAL CALCULI

Slit diaphragm, cylindrical lens patterns of boundaries ascending toward the anode. Glycine buffer, pH 12.0, ionic strength 0.1, potential gradient 6.0 volts per cm., time 7,200 sec.

dicate that uromucoid and the virus inhibitory mucoprotein of Tamm and Horsfall are probably one and the same. In recognition of the original investigations of these workers it is suggested that this material should be referred to as "T&H mucoprotein."

T&H mucoprotein is present in normal urine in remarkably constant quantities, averaging 46 mg. per 24 hours. The ratio of NaCl-insoluble residue to NaCl-soluble proteins in normal urine is thus 1 : 1 (2). In patients with renal calculous disease a marked increase in the urinary content of T&H mucoprotein, with considerable day-to-day variation, was demonstrated. In the present series of 14 such patients the ratio of NaCl-insoluble to NaCl-soluble proteins varied from 2.5 to 14. The 2 patients with bilateral cutaneous ureterostomies were found to have only 18 and 12 mg., respectively, of T&H mucoprotein per 24 hours of urinary excretion. Both had had pyelonephritis and had an average total urinary protein excretion per 24 hours of 460 and 1200 mg., respectively. This finding is in accord with the theory (19) that this NaCl-insoluble material may be a product secreted by the transitional epithelium of the urinary tract. The small quantity present in the urine of these patients is attributed to secretions from the ureters, renal pelves, and possibly the collecting tubules.

The chemical composition of T&H mucoprotein recovered from most of the patients with calculous disease was essentially the same as that recovered from normal individuals. These studies do not afford unequivocal evidence that the mucoprotein is exactly the same material in these two groups of subjects. Tamm has suggested a molecular weight of 7,000,000 for the normal mucoprotein, and considerable alteration in the composition or structure of such a large molecule could easily escape detection by the above methods of analysis.

The alteration in the "solubility" of this mucoprotein by such simple measures as freezing demonstrates the wisdom of Tamm's emphasis on the necessity for technical care in isolating mucoproteins in their native state (8). This effect of freezing is believed to account for the small quantity of fc − 1 previously observed in our own experiments (2), and for the small quantity of the "X" fraction observed by Rigas and Heller (20). The electrophoretic mobility of unfrozen T&H mucoprotein, in the presence of other urinary proteins at pH 8.6, was found to be -7.4×10^{-5} cm.^{2} per volt sec., which is exactly the same at that of fc − 1. When T&H mucoprotein was isolated from other urinary proteins, its mobility was increased. Even in the protein mixture the mobility of this material in the ascending limb of the cell was found to be as much as -8×10^{-5} cm.^{2} per volt sec. in some preparations.

The tendency of T&H mucoprotein to thixotropy is apparently accentuated when the material has been frozen or exposed to temperatures near

0 C. The observation that this material may form a thixotropic gel during the period of thermal equilibration of the electrophoresis cell suggests that the preparatory conditions existing in experiments previously reported (2, 20) may have caused this mucoprotein to appear at two positions in the electrophoretic pattern. The large delta and epsilon boundaries which Rigas and Heller designated "salt plus polysaccharide" may be due in part to immobilization of the T&H mucoprotein as a result of gelation. The fast components (designated fc − 1 in our previous publication and the "X" component by Rigas and Heller) are considered to represent the small "soluble" quantity of T&H mucoprotein which escaped entrapment in the gel.

Tamm, Bugher, and Horsfall has observed that incubation of the mucoprotein at 70 C. resulted in conversion of the original molecule to a more soluble form, which was homogeneous and of reduced sedimentation in the ultracentrifuge (8). Curtain found that treatment with increasing concentrations of urea progressively diminished the axial ratio of this mucoprotein, and that in urea concentrations above 6.5 M the virus-inhibitory titer was also reduced. His urea-treated preparations were much more soluble than the untreated material (11). The present studies have demonstrated that treatment of the mucoprotein at pH 12.3 results in a highly soluble preparation which, to judge from the electrophoretic properties, can be repeatedly duplicated. Whether these three methods for increasing the solubility of the mucoprotein result in disrupting the same bond or bonds remains unknown.

In any event, the interpretation of electrophoretic or chromatographic (21, 22) diagrams of urinary proteins obtained by various methods of recovery (23) should take into account the possible presence of these various degradation products of T&H mucoprotein. The Astrop fraction of urine (24) and the metachromatic urinary "heparin" (25), prepared by methods which involve heating with ammonia, are certain to contain variable quantities of this mucoprotein or its alkaline degradation products, unless the NaCl-insoluble residue is removed prior to heating and treatment at pH 12 or more.

The previously reported affinity of this mucoprotein for calcium (19) has been emphasized by unsuccessful attempts to free calcium from the material with ethylenediaminetetraacetic acid. The possible relationship of this calcium binding activity to the formation of urinary calculi has been discussed (26).

SUMMARY

The NaCl-insoluble residue obtained from the ultrafiltration of urine appears to be a single mucoprotein which is indistinguishable from the virus-inhibitory mucoprotein of Tamm and Horsfall. The quantity of this material is much increased in the urine of patients with renal calculous disease. In the absence of active bacterial infection of the urinary tract, the mucoprotein recovered from such patients was qualitatively similar to that recovered from normal subjects. The methods of analysis, however, are admittedly not sensitive enough to detect alterations in structure or even moderate alterations in composition of the exceedingly large molecules under consideration.

The physical properties of the mucoprotein are altered by such simple laboratory procedures as freezing. This fact emphasizes the meticulous care required to recover this mucoprotein in its native state.

Treatment of this mucoprotein at pH 12.3 results in the formation of a highly soluble degradation product which is consistently recovered even from material that has been denatured by freezing or lyophilization. Recognition of the presence of this degradation product may be of value in studies of the mucoprotein under conditions where denaturization may be unavoidable.

It is suggested that the "X" component of urinary proteins previously observed by Rigas and Heller (20) and the "fc − 1" component observed in this laboratory probably represented a small portion of the total Tamm and Horsfall mucoprotein which remained in suspension. It seems likely that the preparatory procedures, which involved freezing of the urinary proteins prior to electrophoresis, would cause most of this mucoprotein to be insoluble in buffers of 0.1 ionic strength.

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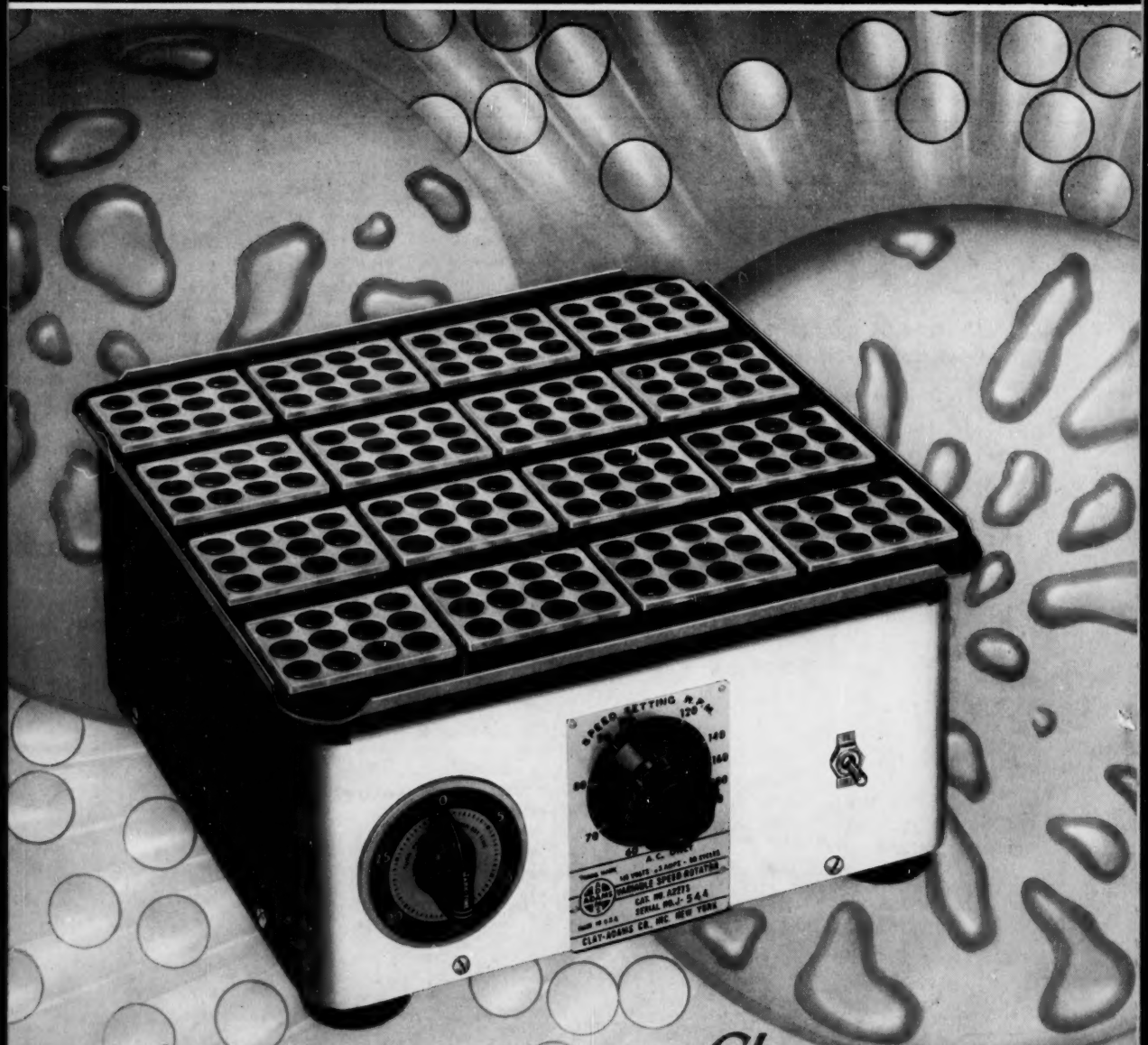

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